The amount of intrahepatic antigen presented by hepatocytes regulates CD8+ mediated T cell responses *in vivo*

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Abbreviations

aa	amino acid(s)
A	adenine or adenosine
AB	antibody
Alb	Albumin (protein)
alb	*albumin* (gene)
ALT	alanine amino transferase
APC	allophycocyanin
APC	antigen presenting cell
bp	base pair(s)
C	cytosine or cytidine
CD	cluster of differentiation
CFSE	carboxyfluorescine succinimidyl ester
Cre	Cre recombinase (fused to ER^{T2})
CTL	cytotoxic T lymphocyte
Cy	cyanine
DAMP	danger associated molecular pattern
DC	dendritic cell
dd	double distilled
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetra acetate
ER	endoplasmic reticulum
ER^{T2}	(tamoxifen inducible) estrogen receptor

et al.	*et alii* (and others)
FC	flow cytometry
FITC	fluorescein isothocyanate
FSC	forward scatter
G	guanine or guanosine
geom.	geometric
GFP	green fluorescent protein
gp_{33-41}	glycoprotein (aa33-41) of LCMV
h	hour(s)
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HIV	human immune deficiency virus
HSC	hepatic stellate cell
hsp	heat shock protein
IFN	interferon
IL	interleukin
i.m.	intra muscular
i.p.	intra peritoneal
i.v.	intra venous
KC	kupffer cells
L	liter
LAG-3	lymphocyte activation gene 3
LCMV	lymphocytic choriomeningitis virus

LN	lymph node
LPS	lipopolysaccharide
LSEC	liver sinusoidal endothelial cell
µ	micro
M	molar
m	milli
MCMV	mouse cytomegalo virus
MEM	modified eagles medium
MFI	mean fluorescence intensity
MHC	major histo compatibility complex
mol/L	mol per liter
min.	minute(s)
n	nano
NPC	non parenchymal cells
o.n.	over night
Ova	Ovalbumin (protein)
ova	*ovalbumin* (gene)
OT-I	Ova specific T cell MHC class I restricted
OT-II	Ova specific T cell MHC class II restricted
PAMP	pathogen associated molecular pattern
PD-1	programmed death receptor 1
PE	r-phycoerythrin
PerCP	peridinin chlorophyll
PRR	pattern recognition receptor

rpm	rounds per minute
RPMI	Roswell park memorial institute
sec.	second(s)
SSC	side scatter
SV40	simian virus
T	thymine or thymidine
Tag	SV40 large T antigen
TCR	T cell receptor
T_{eff}	effector T cell
T_{mem}	memory T cell
T_{reg}	regulatory T cell
TGF	transforming growth factor
TLR	Toll like receptor
v	volume
v/v	volume/volume
vs.	versus
w	weight
WB	western blot
w/v	weight/volume

1. Abstract

The immune system represents a versatile organ that maintains the integrity of the host. However, despite the establishment of immune responses during the acute phase, frequently a failure of immunity is observed leading to a chronic state of infection and persisting pathogen. This phenomenon often takes place during viral hepatitis. CD8+ T cells are crucial mediators of the antiviral immune response, yet, in case of hepatitis C, 70% of patients fail to clear the virus and progress towards chronic viral infection. As a consequence, antigen specific CD8+ T cells acquire an exhausted phenotype and are not capable to eliminate the infection. Studies that aimed to elucidate the causes for an acute failure suggest a local anti-inflammatory effect of the liver itself. Indeed, anti-inflammatory cytokines are continuously expressed within the liver and thus create a tolerogenic environment. However, factors which mediate the outcome of immunity remain elusive. To investigate intrahepatic immunity, an *in vivo* transgenic mouse model was used in this study. This model allows inducible expression of antigen exclusively in hepatocytes. Moreover, by stochastic events, the model enables to establish conditions in which two different amounts of antigen are presented by a defined number of hepatocytes. Thus, the model allows both, the investigation of immune responses towards intrahepatic antigens and the impact of different antigen burden towards responding CD8+ T cells. By using this model to explore CD8+ mediated immune responses within the liver, this work demonstrates a failure of endogenous CD8+ T cells towards antigen expressed by hepatocytes. This failure was also observed, when pro-inflammatory conditions were established coincidentally to antigen expression. Remarkably, intrahepatic immune responses were only achieved upon transplantation of antigen specific CD8+ T cells into antigen expressing mice, as indicated by liver pathology. However, CD8+ mediated clearance of antigen presenting hepatocytes was only accomplished if T cells were exposed to low numbers of antigen presenting cells. Moreover, clearance of low intrahepatic antigen was accompanied by the establishment of protective immunity, while a failure of clearance induced systemic exhaustion and depletion of antigen specific CD8+ T cells. Additionally, T cell exhaustion was also observed, when *in vitro* generated T effector cells were challenged by high numbers of antigen presenting cells. These results provide new insights into the establishment of chronic infections, as they demonstrate a significant role for the concentration of antigen to regulate the course and outcome of CD8+ mediated immunity within the liver.

2. Introduction

2.1 The immune system

The immune system provides a versatile repertoire of cells to combat infections and to maintain the host´s integrity towards threats. These threats include viruses, bacteria, fungi or protozoa infection, but also upcoming tumor cells which could compromise the health of the host. The immune system consists of two different arms: the innate and adaptive immunity (Parkin & Cohen, 2001). All cells of the immune system derive from pluripotent CD34+ hematopoietic stem cells within the bone marrow (Fig. 1 and (Murphy, 2012)). The hematopoietic stem cells have self-renewal capabilities that are maintained upon cellular division (Bryder *et al.*, 2006). Hematopoietic stem cells can differentiate either into a common myeloid progenitor or into a common lymphoid progenitor, respectively. The subsequent fate of these two different progenitors is determined by specific colony stimulating factors that drive the ongoing development of immune cells. All cells that belong to the innate immune system (Fig. 1, right panel) derive from myeloid progenitors, while lymphoid progenitors reflect the precursors of cells which belong to the adaptive immune system (Fig. 1, left panel). One exception are natural killer (NK) cells, as they derive from lymphoid progenitor cells, but are functionally dedicated to the innate immune system (Delves & Roitt, 2000a; Delves & Roitt, 2000b).

Cells of the innate immune system are localized at various sites of the host, where they sense the occurrence of foreign epitopes that reflect a risk of the host´s integrity. The innate immune system responds immediately but unspecifically against any factor that bears a pathogen associated molecular pattern (PAMP) or danger associated molecular pattern (DAMP). However, although the innate immunity constitutes the first line of defense against invading pathogens, it does not provide long lasting protective immunity, since the cells of this arm die during an immune response. A protective immunity is established by cells of the adaptive immune system, namely T and B lymphocytes. These cells mature within the bone marrow (B cells) or the thymus (T cells) and are transported via the blood and lymphatics throughout the whole body (Parkin & Cohen, 2001). Naïve lymphocytes circulate continuously via the blood or lymphatic system until they become activated but do not enter solid tissues (Ando *et al.*, 1994a; Ando *et al.*, 1994b; Bertolino *et al.*, 2001). By inducing apoptosis of infected cells, T lymphocytes are potent effectors against intracellular pathogens, while B lymphocytes release antibodies that protect the host against extracellular pathogens.

During the first response, cells of the innate immune system induce the activation of the adaptive immunity. Thus, the innate immune system can be divided into sub parts: 1. granulocytes represent effector cells that fight against a pathogen, 2. monocytes (dendritic cells (DC), mast cells and macrophages) represent sentinel cells. Due to their capability to take up pathogens by macropinocytosis, sentinel cells can be categorized as phagocytes. Upon phagocytosis, these cells present pathogen derived peptides to cells of the adaptive immune system. Thus, phagocytes reflect a part of the immune system that bridges both, the innate and adaptive immune system (Parkin & Cohen, 2001).

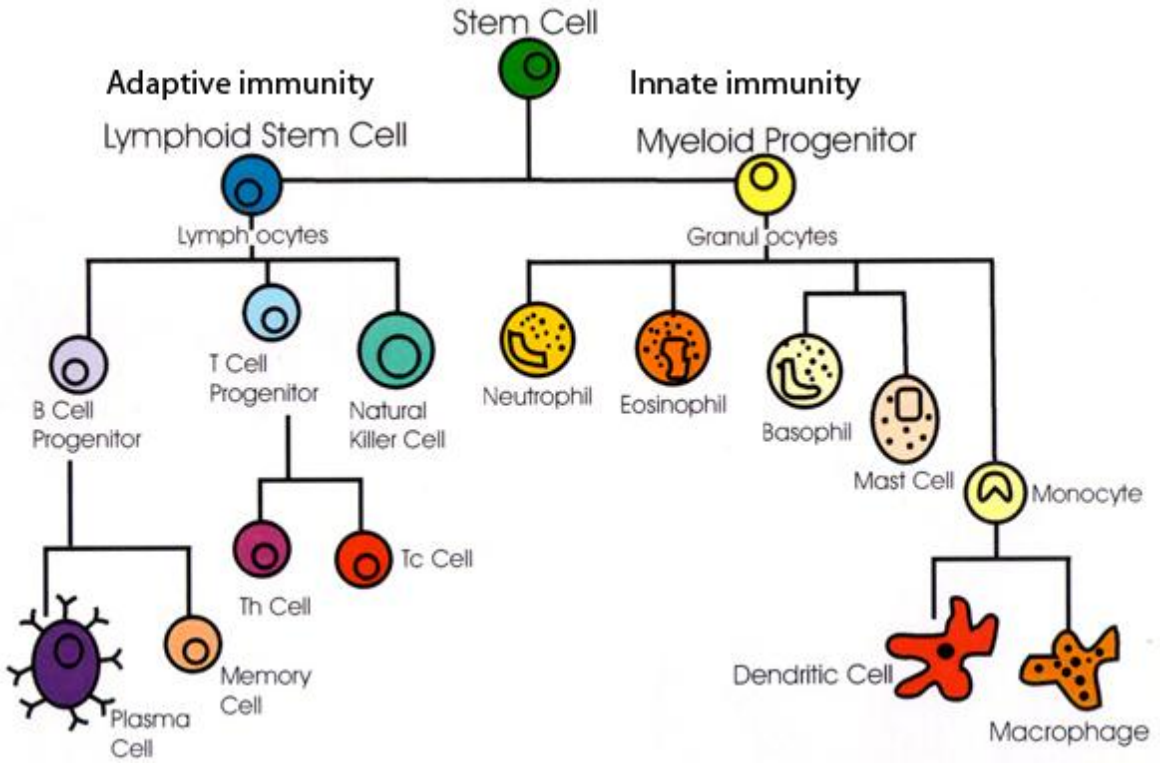

Fig. 1: Overview and development of immune and blood cells. Modified according to textbookofbacteriology.net

2.2 Innate immune responses towards adaptive immunity

In order to immediately protect the host against pathogens, cells of the innate immune system have the capability of discriminating foreign molecules from self. Furthermore, they are localized at positions that represent potential entry ports for pathogens (e.g. mucosa of the respiratory tract). Phagocytes bear pattern-recognition receptors (PRRs) that sense a broad spectrum of PAMPs or DAMPs on microbes (Medzhitov & Janeway, 1997). Such PAMPs are molecules which are conserved on microbes, like lipopolysaccharides (LPS),

lipoteichoic acids and mannans on the cell walls of Gram positive or negative bacteria and yeast, respectively, yet absent on host cells. Upon ligand binding, PRRs induce endocytosis of the pathogen to enhance antigen presentation, nuclear translocation of NF_KB and cell activation (Muzio *et al.*, 2000). As a consequence, activated cells of the immune system release cytokines that induce pro-inflammatory conditions (Parkin & Cohen, 2001). Among these pro-inflammatory cytokines, interleukin-1(IL-1), interleukin-6 (IL-6) and tumor necrosis factor α (TNFα) are master regulators of inflammation which are rapidly released by virtually all cells of the immune system (Malmgaard, 2004). The local increase of these molecules recruits innate effector cells to the site of infection in order to avoid systemic spread of the pathogen. At the same time, DC endocytose pathogens or pathogen derived peptides within the periphery to mediate the transition to the adaptive immunity. DC are professional antigen presenting cells (APC) that acquire an activated phenotype upon endocytosis of pathogen derived peptides. The activation induces both, maturation of DC and expression of surface molecules that allow migration from the inflamed tissue into draining lymph nodes (LN). There, activated DC meet naïve antigen specific T or B cells and present pathogen derived peptides to them, in order to initiate the adaptive immune response (Crotty, 2011; Lanzavecchia & Sallusto, 2001). Responding T cells are activated and acquire an effector phenotype (T_{eff}), characterized by the potential to perform lysis of infected cells. Responding B cells mature into plasma cells that secrete antigen specific antibodies which neutralize the pathogen. However, these defense mechanisms require the access of the immune system to the surface of the pathogen and thus are mainly protective against extracellular pathogens, mostly bacteria. However, by binding on antigens that are presented on the surface of cells, antibodies were also demonstrated to activate NK cells that in turn induce an antibody dependent cellular cytotoxicity (Murphy, 2012).

Intracellular pathogens like viruses in contrast require a rapid protection of the host cell by the induction of molecules that interfere with production of viral RNA or DNA. Those molecules are type I and type II interferons (IFN) that are rapidly produced by infected cells upon recognition of an intracellular pathogen and work in an auto- and paracrine manner. Interferons have anti-proliferative capacity, recruit DC to the site of infection and increase the expression of MHC class I and II molecules on the surface of infected cells (Malmgaard, 2004; Parkin & Cohen, 2001; Sen, 2001). As a consequence, infected target cells present high amounts of virus derived peptides to antigen specific T lymphocytes which were previously primed within draining lymph nodes and subsequently infiltrate the infected tissue. CD8+ cytotoxic T cells specifically lyse target cells bearing the cognate antigen on the surface by either releasing cytotoxic granules into the cytoplasm of infected cells or by inducing a Fas ligand mediated apoptosis of infected cells (Berke, 1995). Upon successful

clearance of pathogens, the majority of effector cells undergo apoptosis. This is necessary to reconstitute immune homeostasis and is mediated by the induction of anti-inflammatory conditions.

2.3 T cell priming within the liver

The onset of adaptive immunity by professional APC requires the integration of multiple signals, namely antigen, co-stimulatory molecules and cytokines. Moreover, the induction of a potent adaptive immune response requires both, effector cells (CD8+ T cells or antibody producing B cells) and CD4+ T helper cells. CD4+ T helper cells interact with the APC and initiate the expression of co-stimulatory molecules to subsequently prime effector cells. This model is called APC licensing (Bennett *et al.*, 1998; Ridge *et al.*, 1998; Schoenberger *et al.*, 1998). The sum of these events takes place within the lymph node and ends in massive clonal expansion of antigen specific T cells (Blattman *et al.*, 2002; Parkin & Cohen, 2001).

Several reports demonstrate that also the liver is a site of T cell activation. Indeed, primary activation of T cells has been shown to take place within the liver itself (Ando *et al.*, 1994a; Ando *et al.*, 1994b; Bertolino *et al.*, 2001; Bowen *et al.*, 2004). This exception of T cell activation is attributed to two parameters, firstly the slow blood flow within the liver and secondly the unique structure of hepatic sinusoids (Fig. 2 and (Bogdanos *et al.*, 2013)). The liver contains a variety of both, professional and non-professional APC, which were demonstrated to be potent activators of CD8+ T cell responses (Bogdanos *et al.*, 2013; Ebrahimkhani *et al.*, 2011). Liver resident DC and kupffer cells (KC) are bone marrow derived immune cells. KC represent a liver specific population of macrophages that perform endocytosis of blood derived antigens and thus function as professional APC. Hepatic stellate cells (HSC) are located closely to vascular sinusoids and in particular to the venules where they endocytose antigens. Moreover, the liver sinusoidal endothelial cell (LSEC) population which is in direct contact to the venous blood has the capability to incorporate and subsequently present antigens. Both, LSEC and KC recognize low concentrations of PAMPs and respond by induction of pro-inflammatory cytokines such as IL-6 and type I IFN (Kern *et al.*, 2010; Wu *et al.*, 2007). The hepatocytes reflect the liver´s parenchym and are non-professional antigen presenting cells. Importantly, hepatocytes express high amounts of MHC class I molecules (Warren *et al.*, 2006) and were also demonstrated to induce T lymphocyte activation *in vivo* (Bertolino *et al.*, 1995; Cebula *et al.*, 2013; Morahan *et al.*, 1989). These findings are remarkable as priming outside of secondary lymphatic tissues

reflects unusual immunological capabilities of the liver according to the classical dogma of immunity.

Yet, although potent immune responses can be induced within the liver, several experimental data suggest that the immune response upon priming by hepatocytes is not as efficient as responses that were induced by professional APC. Bertollino and colleagues demonstrated that T cells are activated towards cytotoxic T lymphocytes (CTL) by hepatocytes *in vitro* but die prematurely (Bertolino *et al.*, 1998; Bertolino *et al.*, 1999). Additionally, *in vivo* data show a failure of immune response upon adoptive transfer of antigen specific CD8+ T cells into transgenic mice expressing the cognate antigen under control of the *albumin* promoter (Limmer *et al.*, 1998). This failure can be overcome by coinciding infection of the recipients with a liver targeting pathogen, like *Listeria monocytogenes* (Limmer *et al.*, 1998). In line with this observation Derkow and colleagues demonstrated that help-independent priming of CD8+ T cells within the liver induces an initial immune response, but fails to maintain this response to eliminate the antigen (Derkow *et al.*, 2011). Moreover, by comparing CD8+ T cell function upon either priming in the liver alone or priming in both, liver and LN, it was reported that liver and LN compete for the priming of T cells (Bowen *et al.*, 2004). Thus, although the liver is rich in cells that are capable of shaping immune responses, the liver also seems to have a potential to regulate the outcome and fate of intrahepatic immunity.

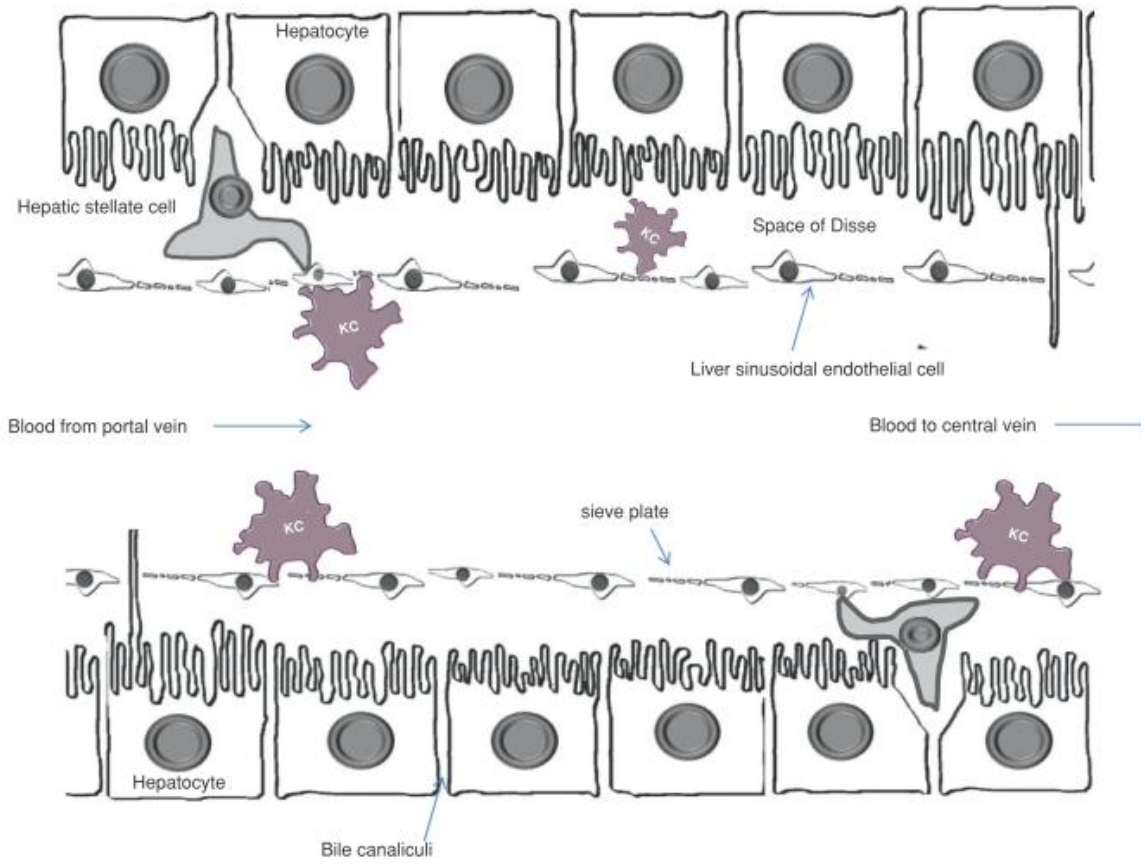

Fig. 2: Microvasculature and anatomy of the liver. Liver sinusoidal endothelial cells (LSEC) shield the hepatic parenchym from the venous blood. To mediate uptake of blood derived metabolites, the LSEC barrier is intersected by pores that allow a fenestration into the space of Disse. These fenestrae additionally allow a transition of migrating lymphocytes from the blood and thus an interaction with antigens presented on hepatocytes. Liver specific APC- hepatic stellate cells (HSC) and kupffer cells (KC)-clear the blood from antigens and present them to migrating lymphocytes. Adapted from (Bogdanos *et al.*, 2013).

2.4 The liver as an immunological organ

The liver is a large and vital organ which fulfills the task as the main metabolic organ within the body. The liver metabolizes carbohydrates, proteins and lipids, but is also responsible for the clearance of toxins and pathogens (Bogdanos *et al.*, 2013; Protzer *et al.*, 2012). While fulfilling this task, the liver is exposed to a high amount of metabolites which are transported into the liver via the blood from the portal vein (Fig. 2). Indeed, the liver receives 30% of the total body´s blood volume per minute (Bogdanos *et al.*, 2013). Additionally, the blood is rich in neo-antigens which are mainly derived from food particles. As a consequence, within the liver an overload of antigenic stimuli is presented to the immune system and an induction of immune response would lead to tissue destruction and metabolic failure. To avoid such tissue destruction, the liver is considered to be rather a tolerogenic than an immunogenic milieu. In particular this becomes evident by the absence of immunity in response to LPS

which is a well-known inducer of pro-inflammatory conditions (Biswas & Lopez-Collazo, 2009; Protzer *et al.*, 2012). This is of biological relevance since the intrahepatic blood also contains high concentrations of endotoxin derived from the commensal gut flora and thus, the induction of an immune response has to be impaired. Moreover, the liver shows a constitutive expression of anti-inflammatory cytokines such as IL-10 or transforming growth factor (TGF). Both molecules were demonstrated to negatively influence T lymphocytes (Holz *et al.*, 2008; Knolle *et al.*, 1995; Tinoco *et al.*, 2009) and thus, naïve T lymphocytes are forced to overcome these suppressive conditions in response to antigens.

2.5 Immune regulation

Immune regulation is a crucial mechanism to control the onset of immune response towards a variety of antigens which are either harmless or auto antigens from the host itself. Especially the onset of immunity towards auto antigens would result in the establishment of immune responses against tissues or organs that would result in a systemic failure of the host. Well known examples for autoimmunity are chronic diseases such as diabetes type I (Roep & Peakman, 2012) or multiple sclerosis (Saxena *et al.*, 2011). Among other immune cells, CD8+ T cells which are specific for auto antigens infiltrate the target tissue and exert cytolytic function, similar as during infection with a pathogen. To avoid autoimmunity, the immune system evolved a mechanism, named negative selection that mediates the deletion of auto reactive lymphocytes during the maturation within both, bone marrow (B cells) or thymus (T cells). During late stages of T cell maturation within the thymus, epithelial cells of the thymic medulla (mTEC) present a broad variety of auto antigens to T cells, that were positively selected and thus express a functional T cell receptor (Shi & Zhu, 2013). Remarkably, mTEC have the capability to present antigens which are only expressed in specialized tissues, e.g. the liver (Klein *et al.*, 1998; Klein *et al.*, 2001). The expression of tissue specific antigens is achieved by the transcription factor autoimmune regulator (Aire) which controls the expression of tissue specific antigens within mTEC (Anderson *et al.*, 2002). Upon positive selection, developing thymocytes interact with self-peptide:MHC complexes presented by mTEC. If a strong ligation of the TCR to the peptide:MHC complex takes place, the responding T cell undergoes activation induced apoptosis to ensure negative selection (Starr *et al.*, 2003; Werlen *et al.*, 2003).

However, some autoreactive T cells escape central tolerance induction within the thymus. This is partially due the fact that not all potential self peptides are expressed by mTEC, e.g. thyroglobulin which is a tissue specific protein (Murphy, 2012). Thus, the suppression or

deletion of T cells that might react against peripheral autoantigens has also to be ensured. Similar to central lymphatic tissues, the fate of autoreactive in the periphery might be deletion or anergy. The regulation is mediated by professional APC that contribute to peripheral negative selection by performing phagocytosis of secreted auto antigens or cellular debris within the periphery (Parkin & Cohen, 2001). Since auto antigens do not activate PRR, APC are only partially activated and migrate to lymphatic tissue where they interact with specific T cells that escaped central negative selection. As this type of antigen uptake does not result in complete activation of APC, they do not express the costimuli and pro-inflammatory which are required for potent activation of the adaptive immune system. As a consequence, self-reactive lymphocytes which respond to autoantigen under non-inflammatory conditions are clonally deleted by the induction of activation induced cell death (van Meerwijk *et al.*, 1997).

In addition to central tolerance induction towards auto reactive T cells and the release of anti-inflammatory cytokines, a third regulative mechanism of immunity has been investigated extensively. This form of regulation is executed by a subset of CD4+ T cells, so called regulatory T cells (T_{reg}). T_{reg} display a CD4+ CD25+ FoxP3+ phenotype (Fontenot *et al.*, 2003; Fontenot & Rudensky, 2005) and exert immune suppressive function towards other lymphocytes in a dose dependent manner (Sakaguchi *et al.*, 2001). Among the T_{reg}, two different populations have been described: on the one hand natural T_{reg} which develop within the thymus, and on the other hand induced T_{reg} which are generated in the periphery in response to anti-inflammatory cytokines, such as Il-10 or TGFβ (Bluestone & Abbas, 2003). To exert suppression, T_{reg} require initial stimulation via the T cell receptor (TCR), indicating an antigen dependent specificity. Yet, once T_{reg} are activated, their suppressive capacity was reported to be also valid for T cells with a different TCR specificity (Sakaguchi *et al.*, 2001; Thornton & Shevach, 2000). Moreover, the antigen dependent activation threshold of T_{reg} requires a much lower concentration of antigen as compared to other lymphocytes. In other words: even low amounts of antigen have been demonstrated to exert suppressive function (Takahashi *et al.*, 1998). Additionally, even low numbers of T_{reg} have been shown to efficiently suppress effector T cell responses (Luth *et al.*, 2008). Thus, T_{reg} reflect a population of lymphocytes that is highly potent to impair the onset of adaptive immune responses. This subset of cells is essential to avoid immune responses against harmless antigens, e.g. food derived peptides, or self-antigens from the host. Since the liver is such a tolerogenic organ, it is not surprising that T_{reg} are abundant within it (Bogdanos *et al.*, 2013) and might be responsible for impairing the onset of a potent immune response within the liver.

2.6 Liver targeting by pathogens

The sum of all intrahepatic influences, namely regulatory cells, lack of supporting CD4+ cells and the immune suppressive cytokine milieu within the liver render this organ susceptible for infections with pathogens. Indeed, several clinically important pathogens target the liver and often establish chronic infections. The most relevant liver targeting pathogens are the malaria sporozoites, hepatitis B and hepatitis C virus (HBV and HCV) which reside mostly within the hepatocytes (Protzer *et al.*, 2012). With about 500 million infected individuals, HBV and HCV are the most common causes of chronic liver infections worldwide (Bogdanos *et al.*, 2013; Rehermann, 2009; Rehermann, 2013).

CD8+ T cells were demonstrated to be crucial mediators to cure acute infections within the liver (Lechner *et al.*, 2000; Maini *et al.*, 1999). Once an infection is cleared, lifelong antiviral immunity is established (Phillips *et al.*, 2010). However, more than 70% of HCV infected patients show a failure of acute viral clearance, progressing towards a chronic state of infection. Evaluation of antigen specific CD8+ T cells revealed an exhausted phenotype, as indicated by expression of inhibitory surface markers, such as programmed death receptor 1 (PD-1) (Radziewicz *et al.*, 2007), T cell immunoglobulin and mucin domain containing molecule 3 (Tim-3) (Golden-Mason *et al.*, 2009; McMahan *et al.*, 2010) or cytotoxic T lymphocyte antigen 4 (CTLA-4) (Nakamoto *et al.*, 2009). The failure of the immune system to clear acute infections is supposed to depend on multiple host factors, including the tolerogenic milieu of the liver itself and the priming of T cells by non-professional APC. On the other hand, stealth mechanisms of various viruses significantly contribute to the failure of immunity, as viruses display strategies to impair classical endogenous MHC class I presentation (Reddehase, 2002). Moreover, viruses were demonstrated to avoid APC function by induction of cell paralysis and impairing natural killer cell (NK) mediated target cell killing (Reddehase, 2002). Especially upon infection of the liver with both, HBV or HCV, a prolonged incubation time of virus is observed. During this time, viremia displays high levels but the immune system tolerates the viral particles. In case of HCV infection, the duration lasts for around 6 weeks until acute immunity is induced, while in HBV infection the tolerant phase can even last for <20 years (Rehermann, 2013). During this time the virus may replicate towards elevated levels and might even acquire mutations that favor to avoid cellular immunity. The consequence of T cell exhaustion and failure of antigen clearance in response to liver infections is a continuous infiltration of immune cells into the liver and release of effector cytokines that induces ongoing tissue destruction. In turn, the liver

becomes fibrotic and even hepatocellular carcinomas (HCC) might develop (Melnikova, 2011). Yet, the fact that acute clearance of virus can be achieved in a small proportion of patients gives hope to develop effective treatments. Thus, an understanding of intrahepatic immune responses towards liver targeting pathogens is essential.

2.7 T cell exhaustion

The failure of acute viral clearance and progression towards a chronic state of infection often correlates with exhaustion of antigen specific T cells. This phenomenon is not restricted to hepatotropic viruses, but rather to a broad variety of viruses, e.g. lymphocytic choriomeningitis virus (LCMV) or human immune deficiency virus (HIV) (Letvin & Walker, 2003; Pantaleo & Koup, 2004; Rehermann & Nascimbeni, 2005; Virgin *et al.*, 2009). When compared to T cell tolerance (induced under non-inflammatory conditions) or T cell anergy (induced by suboptimal T cell priming) which usually occur in response to auto antigen, T cell exhaustion is an unresponsive state of T cells towards pathogens. Exhaustion of T cells can be induced despite the presence of strongly activated APC (Cervantes-Barragan *et al.*, 2012) resulting in an acute T cell response. Exhaustion of antigen specific T cells prevents optimal viral control and is characterized by a variety of phenotypic and molecular changes within the antigen specific T cells (Blackburn *et al.*, 2009; Wherry *et al.*, 2007). Antigen specific T effector cells are initially generated during the early phases of infection, but show a progressive loss of effector function (Zajac *et al.*, 1998). In severe cases even depletion of antigen specific T cells is reported (Wherry *et al.*, 2003). A widely used model to study acute T cell response vs. exhaustion is infection of mice with lymphotropic choriomeningitits virus (LCMV). Depending on the viral strain which is used, adult mice show either viral clearance or progress towards chronic infection (Mueller *et al.*, 2007; Wherry *et al.*, 2003). These two viral strains differ in only two amino acids within the entire genome and none of these mutations affects any of the characterized T cell epitopes (Matloubian *et al.*, 1993). Indeed, the early T cell response against both, acute and chronic LCMV strains, displays similar kinetics, however, only the acute virus is cleared within 8 days upon infection while the chronic strain persists within the host (Wherry *et al.*, 2003). Moreover, clearance of the acute virus results in the development of a potent, protective CD8+ memory population (Paley *et al.*, 2012). However, studying CD8+ T cell responses towards the chronic LCMV strain in mice shows the development and maintenance of an antigen specific progenitor population of CD8+ T cells. This population fails to clear the persistent virus but controls viral spread by induction of homeostatic conditions between viral load and immune response (Paley *et al.*,

2012). The same study reports that such an antigen specific progenitor population is also present in chronic HCV patients and is responsible to control viral replication within the patient. Yet, these cells differ phenotypically from memory cells as they are positive for PD-1, Tim-3 and other exhaustion markers. This phenotype led the authors to the hypothesis that the ongoing effector function in response to the virus might lead to a slow decay of this progenitor cells. In conclusion, the progression towards a chronic state of infection is assumed to be a consequence of T cell exhaustion that takes place in the early phase of infection. In contrast to this hypothesis, clinical observation of chronically infected HIV patients show improved T cell function upon successful reduction of viral replication. As a consequence, antigen specific T cells face lower amounts of antigen and the exhausted phenotype is reverted towards a CTL (Day *et al.*, 2007; Streeck *et al.*, 2008). A clinical study on acutely HCV infected health-care workers indicates a correlation between initial inoculum and outcome of disease. Patients with low inoculum show viral clearance upon acute infection, while patients with high load of viral particles progress towards a chronic state (Thimme *et al.*, 2001). These observations indicate a regulation of T cell responses which is mediated by the amount of antigen itself.

Indeed, *in vivo* models, in which T cells are exposed to low amounts of chronic LCMV, demonstrate the amount of antigen to be the cause of T cell exhaustion (Mueller & Ahmed, 2009; Richter *et al.*, 2012). The authors achieved low amounts of antigen by restricting antigen presentation to DC. During the initial response, enhanced antigen specific T cell clones and improved T cell function were observed. Yet, due to the chosen settings, the virus could not be eliminated and thus, T cells were continuously exposed to sustained antigen and became exhausted. However, these data imply that the quantity of antigen is the consequence of T cell function and thus, decides about either acute clearance or chronic progression. Still, it remains elusive, whether the antigen amount indeed is a regulator of T cell responses.

2.8 Elucidation of intrahepatic immunity

To investigate the onset and progression of T cell responses within the liver, transgenic mouse models have been established in which target antigens are exclusively expressed on hepatocytes (Buxbaum *et al.*, 2008; Derkow *et al.*, 2007; Morahan *et al.*, 1989; Schonrich *et al.*, 1992; Wuensch *et al.*, 2006). The disadvantage of these models is that the immune system considers the target antigen to be `self ´ as it is expressed from birth onwards and thus, is tolerated by the immune system (Delves & Roitt, 2000a; Delves & Roitt, 2000b; Miller

& Basten, 1996). A different approach to study intrahepatic immunity is either the transfer of hepatotropic viruses that express the target antigen, e.g. adenovirus or the hydrodynamic injection of DNA that is accompanied by hepatocyte specific uptake (Huang *et al.*, 2012; Stabenow *et al.*, 2010; Wohlleber *et al.*, 2012; Yang *et al.*, 2002). However, both protocols result in only a partial transduction of hepatocytes and thus, only affect a small amount of antigen presenting cells. Additionally, these approaches do not reflect clinical cases since the immune system is capable to clear both, adenovirus and the injected DNA which differs from infection with HBV and HCV. Furthermore, viral and non-viral gene transfers were shown to be accompanied by severe inflammation and liver damage which might mask immune responses towards antigens presented on hepatocytes (Hartman *et al.*, 2007; Racz *et al.*, 2011). Taken together, there is an urgent need for animal models which exhibit the following characteristics: 1. allow the investigation of immune responses towards antigens exclusively present on hepatocytes; 2. prevent stimulation of innate immunity to achieve specific interactions between CD8+ T cells and the target cell; 3. provide the opportunity to specifically analyze factors and pathways that lead to the induction of T cell anergy, tolerance or exhaustion as often reported within several infectious diseases.

2.9 Aim of the study

The liver has been demonstrated to be a potential niche for clinically relevant pathogens to establish infections (Protzer *et al.*, 2012). Despite the demonstration of CD8+ T cells to be essential regulators within intrahepatic immunity, infection with HCV often leads to viral persistence and exhaustion of antigen specific T cells. The factors that determine acute viral clearance vs. chronic progression are thought to depend on the tolerogenic milieu of the liver, but largely remain elusive. Studies have already suggested a correlation between initial inoculum of antigen and outcome of immune responses (Thimme *et al.*, 2001).

The focus of this study is the characterization of determinants that govern the efficiency of CD8+ T cell mediated immune responses within the liver.

By using a double transgenic Ova x CreER^{T2} mouse model that mimics *de novo* expression of the antigen Ovalbumin exclusively in hepatocytes (Sandhu *et al.*, 2011), the induction of endogenous CD8+ T cell responses towards antigen presenting hepatocytes was investigated. This model is a sterile model of infection as the expression of antigen is restricted to hepatocytes and does not result in the activation of the innate immune system (Sandhu *et al.*, 2011). By providing pro-inflammatory conditions coincidental to *de novo*

expression of antigen it was focus of investigation whether CD8+ mediated immunity can be established. Moreover, the use of antigen specific CD8+ in allogenic transplantation experiments aimed to investigate the influence of different antigen quantities towards intrahepatic CD8+ T cell responses, as well as the subsequent fate of provided antigen specific CD8+ T cells. Additionally, it was compared if the phenotype of antigen specific T cells (naïve vs. pre-activated) influences the onset and progression of liver immunity.

The controlled investigation of CD8+ T cell responses towards the target cells of hepatotropic viruses in absence of side effects by the innate immune system would significantly contribute to the understanding of early events upon liver infection. Moreover, elucidation of mechanisms that prevent acute clearance of antigen and induce T cell exhaustion might reveal new aspects of therapeutic treatments that could improve the function of CD8+ T cells under exhaustive conditions.

3. Results

3.1 Hepatocyte specific expression of Ova within transgenic mice

3.1.1 Generation of double transgenic mice to achieve hepatocyte specific antigen expression

The transgenic mouse model Ova x Cre (Cebula *et al.*, 2013; Sandhu *et al.*, 2011) allows studying immune responses towards hepatic antigens without contribution of the innate immune system. The model is based on heterozygous Ova mice which encode a fusion protein comprising three components: 1. hsp 73 capturing N-terminal viral J domain of SV40 Tag (cT_{77}); 2. 108 aa residue of Ova (aa246-353) with well characterized Kb- and Ad/b-binding epitopes (specifically recognized by OT-I and OT-II cells) and 3. a GFP reporter protein. The fusion gene is integrated in reverse orientation to the *Rosa26* promoter and thus, is inactive. Activation of the transgene can be achieved by expression of Cre recombinase which will recombine the inversely orientated *loxP* sites which flank the transgene (Fig. 3A). Tissue specific activation is achieved by mating the Ova mice to $AlbCreER^{T2}$ transgenic animals that express a tamoxifen (Tam) inducible $CreER^{T2}$ recombinase (abbreviated with Cre in the following) under the hepatocyte specific *Albumin* promoter (Schuler *et al.*, 2004). Antigen expression in double transgenic Ova x Cre mice is induced by treatment with Tam (Fig. 3A upper panel and (Cebula *et al.*, 2013)). Upon application of Tam, Cre translocates into the nucleus of hepatocytes and mediates the recombination, resulting in reversion of the antigen cassette and activation of expression (Fig. 3A). The Cre mediated recombination is reversible as long as Tam is present within the blood circulation, thus both an antisense and a sense mRNA of *ova* are co-expressed (Fig. 3A). Eventually, when a pulse of Tam is applied and finally cleared, the orientation of *ova* becomes fixed leading to theoretically 50% of hepatocytes expressing the antigen (ON state), whereas 50% remain in the OFF state (Fig. 3 B2). Both orientations of *ova* can be determined by qRT-PCR, using specific primers that specifically generate a PCR product in the ON state (primer pair 1a/4a) or in the OFF state (primer pair 3a/4a), respectively (Fig. 3A and (Cebula *et al.*, 2013)).

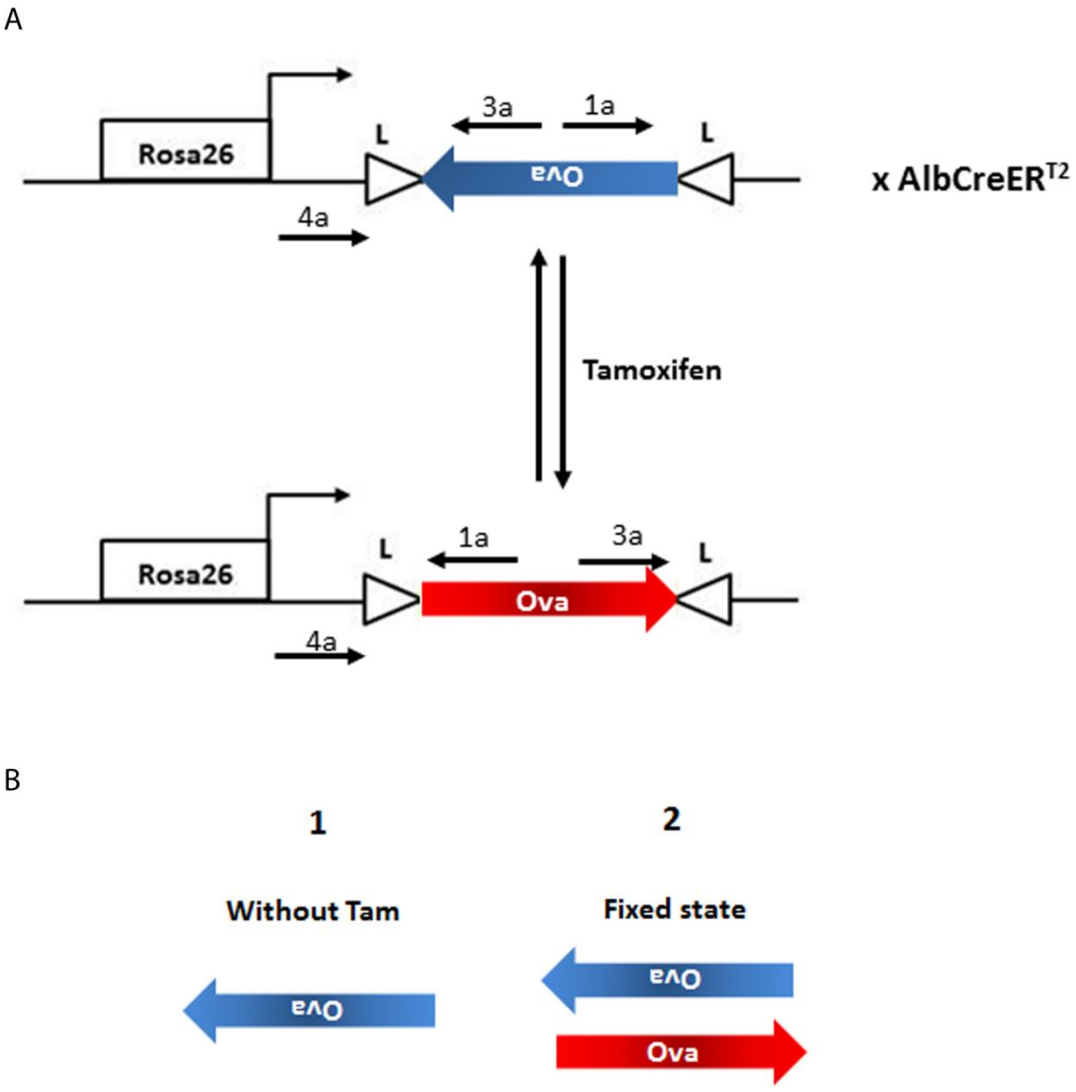

Fig. 3: Overall scheme of hepatocyte specific antigen expression in Ova x Cre mice. (A) In single transgenic Ova mice the antigen is targeted in the ubiquitously active rosa26 locus and flanked by inversely orientated *loxP* sites. As the orientation of *ova* is in antisense direction, the epitope is not expressed. Ova mice are bred to AlbCreERT^{2} (Cre) transgenic mice in which the tamoxifen (Tam) inducible CreER^{T2} recombinase is under control of the hepatocyte specific *albumin* promoter. CreER^{T2} is present within the cytoplasm and requires a Tam mediated conformational change to translocate into the nucleus and induce recombination of *loxP* sites. Thereby, the sense orientation of *ova* is induced and Ova is expressed. The position of primer combinations 1a/4a or 3a/4a is used to exclusively detect one of both possible *ova* orientations by qRT-PCR as indicated. (B) Due to the inverted orientation of *loxP* sites, Tam induced recombination events can occur more than once, thus leading to an ON state (sense) or OFF state (antisense), as long as Tam is present. Once Tam is metabolized, the orientation of *ova* becomes fixed, theoretical leading to 50% of hepatocytes within the ON state and 50% of hepatocytes within the OFF state.

3.1.2 Ova x Cre mice express different amounts of antigen in a Tamoxifen dependent manner

To analyze the status of antigen expression Ova x Cre mice were sacrificed and liver tissue was isolated 14 days upon Tam application. Previous data showed that at this time point

Tam is cleared from the circulation (data not shown). qRT-PCR was used to quantify the mRNA levels of *ova*. The primer combination 1a/4a exclusively gives rise to a PCR product of *ova* within the ON state (Fig. 3). The expression levels of *ova* were related to *albumin* which is exclusively expressed in hepatocytes and served as a cell type specific internal control. Quantitative analysis reveals absence of a specific product in Ova single transgenic mice which are deficient for Cre (Fig. 4A). Ova x Cre mice that received Tam, display a detectable product (Fig. 4A). In agreement with previous data (Cebula *et al.*, 2013) a 5fold reduced but still significant expression of *ova* was also found in Ova x Cre mice without Tam treatment (Fig. 4A). This basal expression confirms reports on corresponding RosaLuc x AlbCreER^{T2} mice in which *luciferase* is expressed in a similar expression cassette (Sandhu *et al.*, 2011). The constitutive expression in the absence of Tam is attributed to the high expression level of CreER^{T2} protein that might induce recombination even in the absence of Tam. Such a Tam independent Cre activity was previously observed during the embryonic and postnatal development in an independent model (Imayoshi *et al.*, 2006).

The qRT-PCR of Tam induced and non-induced Ova x Cre mice two weeks after single Tam application showed two different expression levels of *ova* mRNA within the liver of Ova x Cre mice which would correspond to two different amounts of expressed antigen. The expression of antigen per cell is similar in Tam treated and non-treated Ova x Cre mice, since only heterozygous Ova mice were used within the experiment. Thus, a single hepatocyte can only be in either the ON or the OFF state. Thus, the differences of antigen expression have to be considered on whole tissue level but not on cellular level.

According to the theoretical calculation, the induced expression of *ova* should correspond to a maximum number of 50% hepatocytes within the liver. Since 50% of hepatocytes are expected to express the antigen upon Tam administration, the basal expression is expected to relate to not more than 10% of hepatocytes. As the expressed antigen is a fusion of Ova to GFP (Sandhu *et al.*, 2011) flow cytometry was performed on hepatocytes from Tam treated and untreated Ova x Cre mice to quantify the amount of antigen expressing cells (Hillebrand and Wirth, unpublished). However, GFP positive cells were not detected. Using whole cell extracts in western blot experiments also failed to detect the protein (data not shown). This might be due to both, a low expression of *ova* transcript which is 100fold less than actin (Cebula *et al.*, 2013) and the accelerated decay of protein due to the viral J-domain within the fusion protein (Sandhu *et al.*, 2011). Thus, sensitive methods as RNA detection seem to be more applicable to quantify the amount of *ova* expression within hepatocytes of Ova x Cre mice. However, detection of antigen by whole mount RNA *in situ* hybridization (WISH) on

liver tissue failed, as the probe could not enter the hepatocytes due to the capsule of the liver (data not shown).

Thus, qRT-PCR is the only method to quantify the amount of antigen expressing hepatocytes. To determine the frequency of antigen expressing cells, the amount of *ova* transcript was calculated in relation to Ova mice in which 100% of cells are in the OFF state. Therefore the primer combination 3a/4a (Fig. 3A) which exclusively gives rise to the antisense product of *ova* was used to determine the expression of antigen. The expression values of the Ova single transgenic mice were considered to correspond to 100% antisense expression. The values of antisense expression in Ova x Cre mice were lower if compared to the 100% non-expressing control, reflecting the basal activation of *ova* in these mice. The abundance of antisense transcript within the liver of Tam treated Ova x Cre mice was reduced by ~50% if compared to single transgenic Ova mice (Fig. 4B). These values were calculated as decreased percentage of antisense expression which gives rise to 54% of hepatocytes expressing *ova* upon Tam treatment and 14% of hepatocytes expressing *ova* in uninduced Ova x Cre mice (Fig. 4C).

Taken together, Ova x Cre mice show a Tam dependent expression of antigen, allowing the investigation of CD8+ mediated immune responses towards two different antigen loads, low and high (corresponding to Ova x Cre mice –Tam and Ova x Cre mice +Tam, respectively (Fig. 4D)).

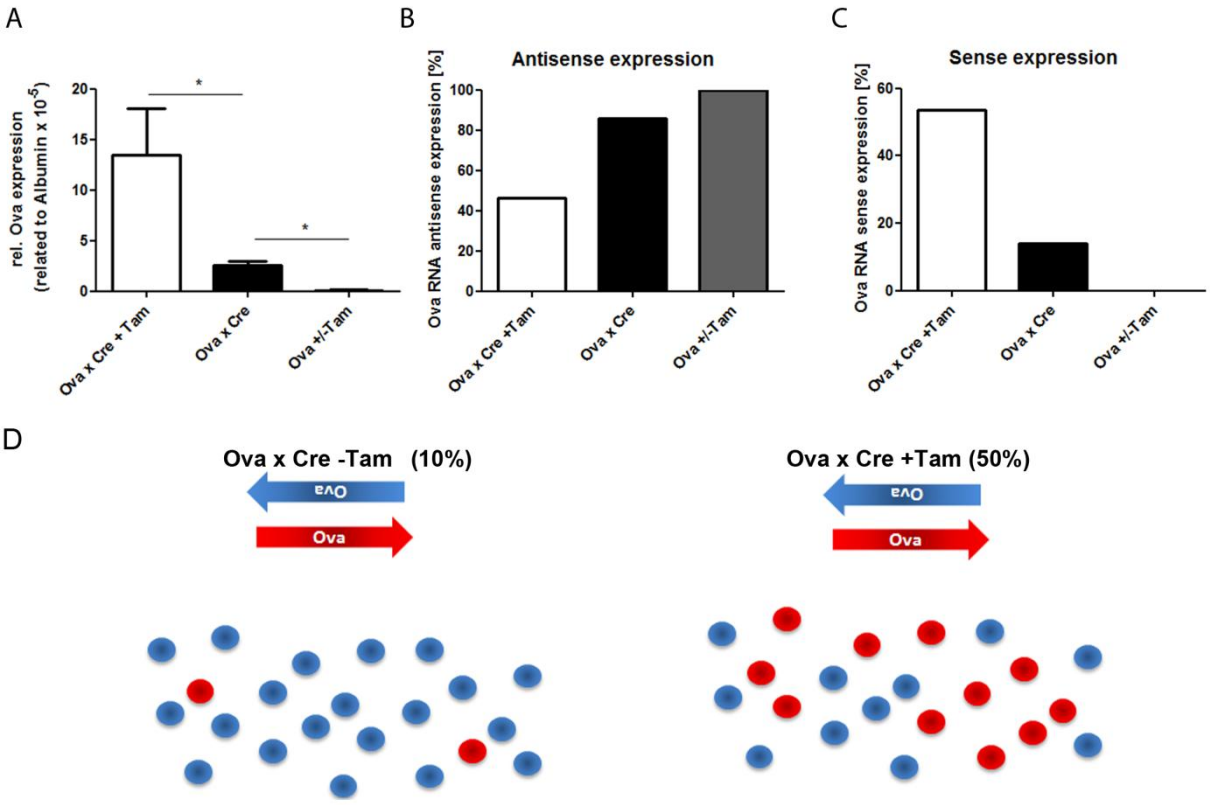

Fig. 4: Evaluation of the antigen expression within the liver by qRT-PCR. Indicated mice were either treated with a single dose of 50 µg Tam or left untreated. 14 days later mice were sacrificed and liver tissue was isolated to purify the total mRNA by column preparation (see: RNA purification from liver tissue). (A) To investigate the antigen expression within the liver, qRT-PCR using the primer combination 1a/4a was performed on 250 ng total cDNA. Expression of *ova* was related to *albumin* expression as internal control. Analysis reveals an expression of *ova* in Tam treated Ova x Cre mice which is 5fold higher than the basal expression in non treated Ova x Cre mice. n≥4; p≤0,05 using Mann-Whitney test. (B) To evaluate the percentage of hepatocytes expressing the antigen, qRT-PCR was performed on cDNA derived from total liver tissue using the primer combination 3a/4a specifically for the antisense product (compare to Fig. 3). The relative expression of an Ova single transgenic mouse was set to 100% and used to calculate the percentage of antisense expression in Ova x Cre ±Tam mice (n>/=2). (C) Using the values of antisense expression from (B), the percentage of expressing hepatocytes was calculated. (D) Scheme of different antigen burden in the liver of Ova x Cre mice. 10% (left panel) or 50% (right panel) of hepatocytes express the antigen dependent on Tam application. Antigen expressing hepatocytes and *ova* sense orientation are depicted in red. The expression per cell is similar in both conditions, but the amount of antigen presenting cells within the liver differs, dependent on Tam treatment.

3.2 Immune competence of Ova x Cre mice

3.2.1 Central and peripheral tolerance are absent within Ova x Cre mice

Molecular characterization of the underlying mouse model Ova x Cre by qRT-PCR revealed basal intrahepatic Ova expression that occurs even in the absence of previous Tam application. The basal expression of Ova might be due to leakiness of $CreER^{T2}$ which has previously been demonstrated to occur during embryogenesis in an independent mouse model (Imayoshi *et al.*, 2006). Thus, it was asked if Ova was also expressed within the thymus of Ova x Cre mice prior to activation and might induce central tolerance towards the

developing T cells (Miller & Basten, 1996). The induction of central tolerance towards Ova specific T cells would depend on two events: 1: The transcriptional activity of Aire would lead to thymic expression of $CreER^{T2}$ that is under control of the *albumin* promoter. 2. The leakiness of $CreER^{T2}$ that is observed within the liver might also be valid within thymic epithelial cells. As a consequence, the antisense cassette of *ova* might be recombined and the antigen would be expressed by the ubiquitous *Rosa26* promoter in thymic cells in absence of Tam.

Recently, a tetracycline inducible model for Ova expression within the skin which was characterized by a certain leakiness that revealed an increased development of T_{reg} and reduction of CD4+ T_{helper} cells in presence of Ova specific CD4+ T cells (OT-II). Accordingly, the induction of T_{reg} and reduction of T_{helper} cells can be considered to be sensitive indicators for thymic expression of Ova antigen (Rosenblum *et al.*, 2011).

To evaluate the unintended presence of thymic Ova expression, Ova x Cre mice were bred to OT-II mice. T cells were isolated from thymus, spleen and liver draining LN of uninduced OT-II x Ova x Cre mice. Both, OT-II x Ova mice and single transgenic OT-II mice served as controls, to proof the absence of Ova expressed from an antisense transcript in the thymus. Evaluation of the CD4+ frequencies showed no significant differences among the groups (Fig. 5A and data not shown). Gating on CD4+ CD25+ FoxP3+ T_{reg} within the thymus showed that the frequencies of T_{reg} were not increased if compared to the controls (Fig. 5B). The fact that the T_{reg} population is not increased, excludes both, intrathymic expression of Ova and additionally expression of the antisense transcript that might induce central tolerance.

Yet, the leaky expression within the liver, as confirmed by qRT-PCR (Fig. 4A) might result in the development of peripheral tolerance. To investigate the mechanisms of peripheral tolerance induction, T_{reg} were investigated in both spleen and liver draining lymph node. No increased frequencies of these regulatory cells that might impair the onset of intrahepatic immunity mediated by CD8+ T cells were detected in the uninduced state (Fig. 5C and D). This suggests that the leaky expression of antigen in the liver is ignored by the immune system. Moreover, treating triple transgenic OT-II x Ova x Cre mice with Tam did also not result in increased T_{reg} frequencies within the liver or spleen if compared to both, uninduced triple transgenic mice or OT-II x Ova control mice (data not shown). In addition by inducing Ova expression within the liver of triple transgenic OT-II x Ova x Cre mice did not result in increased frequencies of CD4+ T cells if compared to both, untreated triple transgenic mice and OT-II x Ova controls (data not shown). Since CD4+ T cells are only activated by antigen within the context of MHC class II molecules, these results confirm a previous report that

hepatocytes do not express MHC class II molecules under physiological conditions (Herkel *et al.*, 2003). Moreover, these results indicate that the antigen is not secreted within the present Ova x Cre mouse model.

Taken together, leaky expression of Ova antigen within the thymus can be excluded by using a triple transgenic OT-II x Ova x Cre system. This indicates a strict regulation of antigen only within the liver. Moreover, by absence of increased T_{reg} cells the presence of peripheral tolerance within the liver can be excluded.

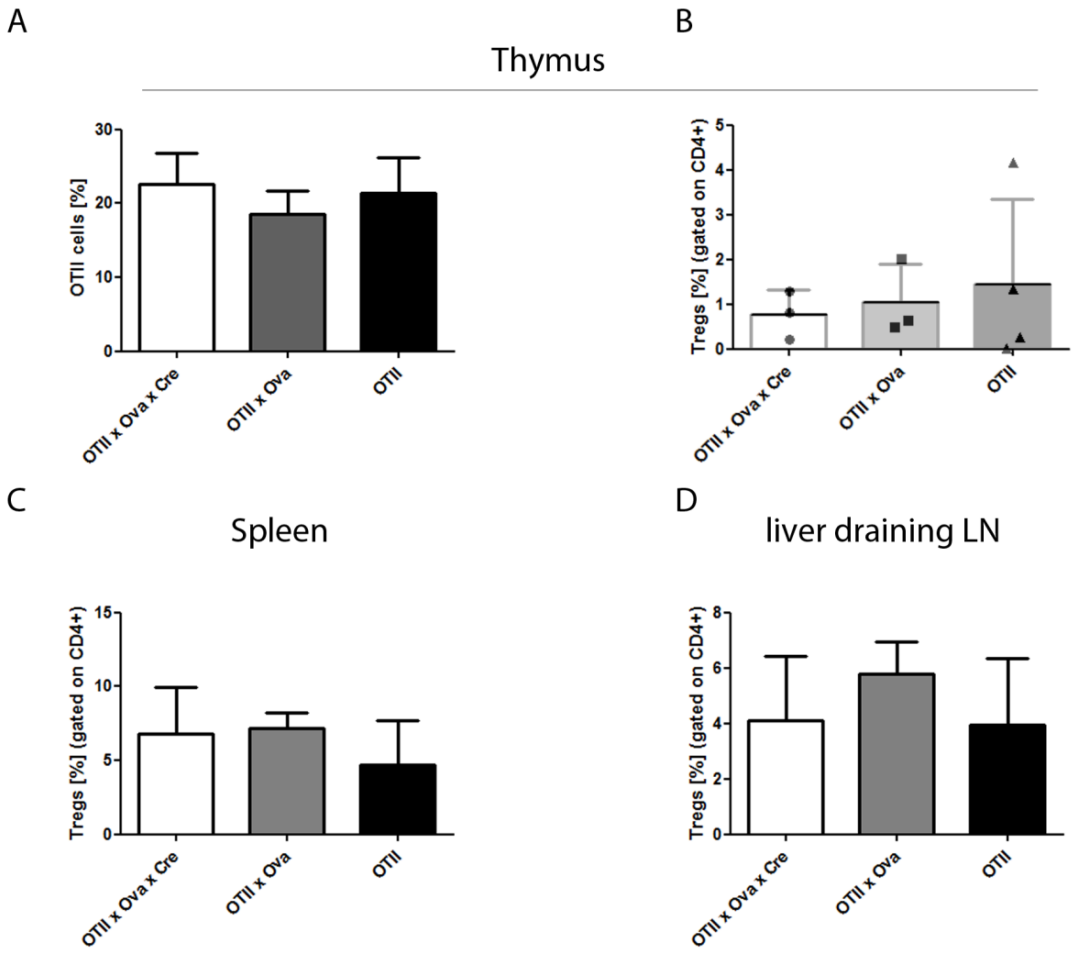

Fig. 5: Absence of central or peripheral tolerance in Ova x Cre mice. To exclude central or peripheral tolerance mechanisms due to the leaky expression of Ova, Ova x Cre mice were bred to transgenic bearing CD4+ T cells specific for Ova. Mice were not treated with Tam and T cells were isolated from thymus (A and B) or spleen (C) and liver draining lymph node (LN) (D). Isolated T cells were stained for CD4, CD25 and FoxP3 to investigate the percentage of Ova specific T_{reg} by flow cytometry. Shown is the mean of n>3 mice per group ±SD. Due to the high variation in (B) bars are overlaid with the frequencies of T_{reg} in the thymus of individual mice.

3.2.2 Endogenous CD8+ T cells in Ova x Cre mice are tolerated towards Ova

Upon confirming the absence of central and peripheral tolerance mechanisms in Ova x Cre mice it was asked if a CD8+ T cell mediated immune response was established towards activation of Ova presentation exclusively in hepatocytes. Thus, Ova x Cre mice were treated with Tam or left uninduced to achieve both, high and low expression of antigen within the liver. 14 days later mice were sacrificed and liver resident CD8+ T cells were investigated for antigen specificity by flow cytometry. This was achieved by staining isolated T cells with a tetramer which presents the Ova derived peptide SIINFEKL and is recognized by the T cell receptor of antigen specific CD8+ T cells.

Interestingly, Ova specific CD8+ T cells were not detected in both, Tam treated or nontreated Ova x Cre mice (Fig. 8 Ova x Cre +Tam and data not shown). This confirmed previous characterization of the Ova x Cre model (Hillebrand and Wirth, unpublished). However, when vaccinating the mice by intramuscular application of a plasmid encoding Ova, the development of SIINFEKL specific CD8+ T cells was observed (Hillebrand, Cebula and Wirth, unpublished). This indicates that the induction of Ova expression on hepatocytes per se does not give rise to antigen specific CD8+ T cells. This suggests impairment of the development of antigen specific CD8+ T cells.

3.2.3 Liver inflammation by adenovirus

The lack of tetramer positive T cells upon inducing antigen expression by Tam led to the suggestion that the tolerogenic milieu of the liver impairs the development of functional Ova specific CD8+ T cells and results in a failure of potent immune response. The observed failure of T cell development might be due to the fact that in Ova x Cre mice induction of antigen expression is not accompanied with the activation of the innate immune system by pathogen associated molecular patterns. Thus, the onset of Ova expression in Ova x Cre mice can be considered to occur in the absence of the classical hallmarks of infection such as inflammation induced by viral nucleic acids or cell wall debris of microbes (Delves & Roitt, 2000b; Parkin & Cohen, 2001). The absence of pro-inflammatory conditions upon antigen expression rather reflects a sterile infection and might compromise the development of a functional T cell response. Thus, it was investigated if activation of the innate immune system coincidental to antigen expression would allow establishing a functional T cell response, thereby complementing the sterile model Ova x Cre. To test this hypothesis, pro-inflammatory conditions were established within Ova x Cre mice by a simultaneous infection with a recombinant non-replicating adenovirus. Adenoviruses have been shown to be

preferentially hepatotropic *in vivo* (Hegenbarth *et al.*, 2000; Sprinzl *et al.*, 2001; Stabenow *et al.*, 2010; Wuensch *et al.*, 2006; Wuensch *et al.*, 2010). Moreover, adenoviruses have the advantage to be non-replicating *in vivo* if the *E1* region is deleted and thus can be either used as vectors to deliver genes (Arbuthnot *et al.*, 1996; Bao *et al.*, 1996) or to induce an inflammation without establishment of a persistent state.

To induce CD8+ mediated T cell responses within the liver, Ova x Cre and BL/6 mice were treated with Tam or left untreated. Simultaneously to Tam application mice were infected i.v. with 1 x 10^{10} pfu to achieve both, Ova expression and stimulation of the innate immune system at the same time. To monitor the route of infection, a recombinant adenovirus encoding luciferase and GFP (AdLucGFP) was used. The animals were monitored by *in vivo* bioluminescence measurement to detect virus encoded luciferase (for detailed description see 7.5.1.2 *In vivo* bioluminescence imaging). *In vivo* bioluminescence imaging on day 3 upon infection confirmed successful infection and indicated liver tropism of the adenovirus. All mice that were infected with virus showed a specific signal for luciferase in particular in the center of the body whereas no luciferase was detected in uninfected mice (Fig. 6A). Following the kinetics of infection by bioluminescence *in vivo* imaging showed a peak of signal on day 7 in all groups that declined by day 12 (Fig. 6B). Uninfected mice remained negative for luciferase over the whole time of experiment. This kinetic indicates a transient infection of the liver as expected from a replication-incompetent vector.

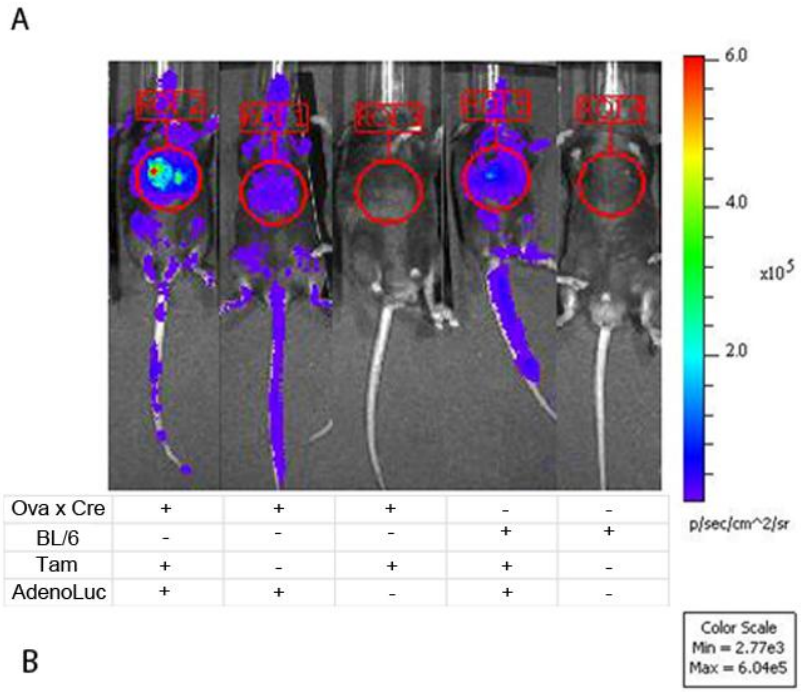

Fig. 6: Route of adenoviral infection. Ova x Cre or BL/6 mice were infected with 1 x 10^{10} pfu AdLucGFP on day 0. Ova expression was induced by application of 8 mg Tam on day -2 and -1. To confirm adenoviral infection *in vivo* bioluminescence measurement was performed on day +3, +7 and +12 upon infection. (A) Representative images of individual mice on day 3 (n= 3 mice/ group). (B) Kinetic of luciferase expression upon infection of mice as determined by quantification of *in vivo* bioluminescence measurement on indicated time points (n=3 mice/group).

To confirm the liver tropism of adenovirus and the induction of CD8+ mediated liver pathology, serum alanine amino transferase (ALT) levels were monitored on different stages relative to infection. The enzyme ALT is preferentially located within the cytoplasm of

hepatocytes and is released into the blood upon hepatocyte damage. Thus, ALT is often used in the clinics to determine the health status of the liver during infection (Rehermann, 2009; Stabenow *et al.*, 2010).

All mice displayed physiological levels of ALT prior to infection, as indicated by serum values below 40 U/L (Fig. 7). In line with other reports (Huang *et al.*, 2012; Wohlleber *et al.*, 2012), increased levels of ALT were detectable in all groups of mice that were infected with virus on day 7 upon infection. Liver damage was monitored until day 14 when the experiment was finalized and mice were sacrificed. Analysis of ALT levels at this timepoint indicated ongoing liver pathology (Fig. 7). In comparison, uninfected control mice did not show elevated ALT levels, irrespective of Tam treatment (Fig. 7). Taken together, the data confirm that infection with adenovirus induces a specific intrahepatic immune response, suggesting the induction of pro-inflammatory conditions by adenovirus.

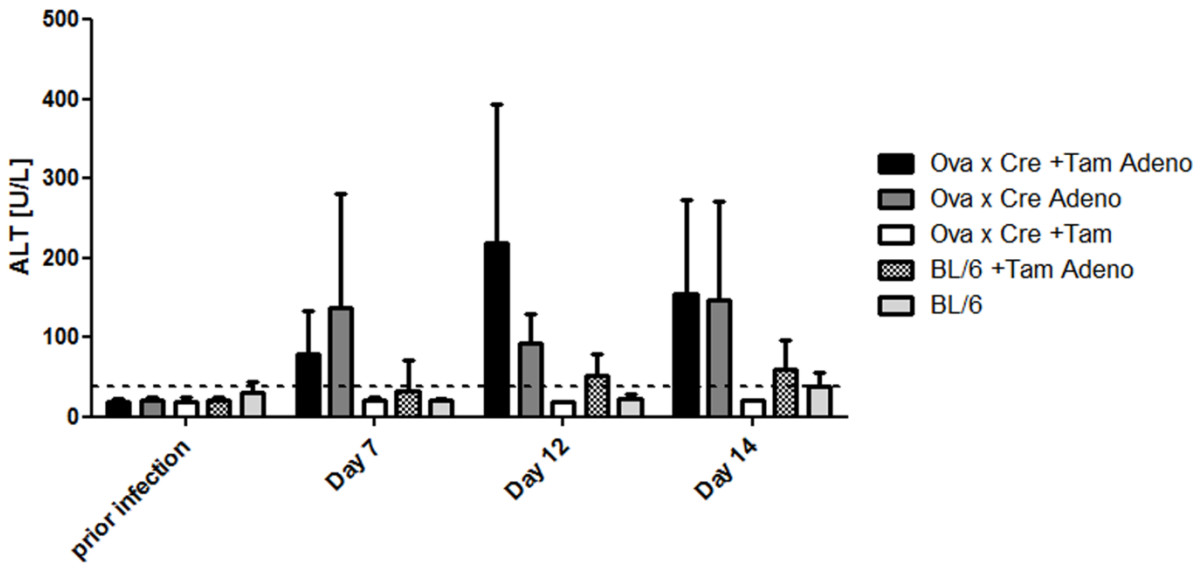

Fig. 7: Adenoinfection specifically induces liver pathology. Ova x Cre or BL/6 mice were infected with 1 x 10^{10} pfu AdLucGFP on day 0. Simultaneously to infection Ova was induced by application of 2 x 8 mg Tam on day -2 and -1. To evaluate immune mediated liver damage, serum ALT levels were measured at the indicated time points. Mean of n=3 mice per group ±SD. The threshold of physiological ALT values (<40 U/L) is depicted by the dashed line.

3.2.4 Adenoviral infection induces CD8+ T cells, but does not lead to Ova specific T cell clones

Having confirmed that adenoviral infection results in liver damage, it was investigated if this observed immune pathology was mediated by CD8+ T cells. In particular, it was of interest to

investigate if these conditions supported the development of Ova specific CD8+ T cells. Hence, animals were sacrificed on day 14 upon infection. Analysis of CD8+ T cells by flow cytometry showed increased CD8+ frequencies in the liver of all groups of mice that were infected with AdLucGFP (Fig. 8). The uninfected control groups displayed ~3-4fold less CD8+ T cells within the liver and were comparable to non treated BL/6 animals. However, staining with SIINFEKL tetramer showed absence of CD8+/tetramer double positive cells, indicating that no Ova specific T cells were induced (Fig. 8). These data suggest that induction of Ova expression coinciding with an adenoviral infection induces the generation and activation of CD8+ T cells which leads to hepatitis. Yet, the absence of Ova specific CD8+ T cells suggests that hepatitis is mainly mediated by T cells specific for adenoviral antigens. Together this implies that also these conditions can not overcome tolerance of Ova specific CD8+ T cells.

It was speculated that a competition for epitopes might have been established among T cell clones during the acute response against adenovirus. As a consequence, T cells specific for Ova might have been outcompeted. To overcome the potential competition, mice were treated with Tam again on day 14 upon infection with adenovirus, i.e. a time point when the virus had been cleared from the hepatocytes. However, analysis of T cell phenotypes upon second induction of antigen expression revealed the absence of SIINFEKL-tetramer positive CD8+ T cells (data not shown). Similar results as observed for infection with adenovirus were detected, when a replication competent mouse cytomegalovirus (MCMV) was used. If compared to adenovirus, MCMV was expected to establish a longer lasting infection. Elevated ALT levels were detected in all groups of mice that were infected by MCMV, while the controls remained healthy (data not shown).

Alternatively to infection with viruses, it was investigated whether an unspecific transient liver damage induced by i.p. application of CCl_4 resulted in development of Ova specific CD8+ T cells, which were primed by cross-presenting APC. Although increased ALT levels (≥1500 U/L) were observed within 24 h upon CCl_4-treatment, Ova specific CD8+ T cells could not be detected. Finally, unspecific pro-inflammatory conditions were induced by PolyI:C, LPS and R848. These synthetic agents are known to activate Toll-like-receptors (TLR) 3, 4 and 7/8, respectively (Alexopoulou *et al.*, 2001; Hemmi *et al.*, 2002; Matsumoto *et al.*, 2002; Poltorak *et al.*, 1998; Shimazu *et al.*, 1999). Despite an onset of inflammation within the liver via the activation of TLR´s neither liver damage, nor development of Ova specific CD8+ T cells were observed (data not shown). This suggests an onset of intrahepatic immune response. However cross-presentation of Ova and development of Ova specific CD8+ T cells was not achieved (data not shown).

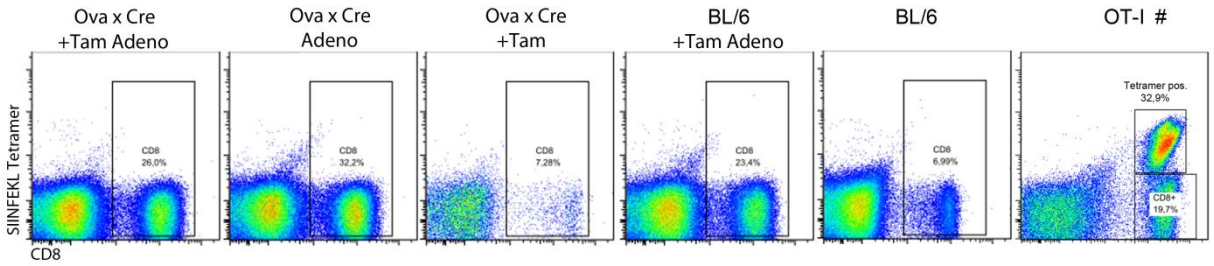

Fig. 8: Adenoviral infection in mice induces CD8+ T cell immunity without development of Ova specific CD8+ T cells. Intrahepatic CD8+ T cells were isolated from AdLucGFP infected Ova x Cre or BL/6 mice on day 14 upon infection. CD8+ T cells were analyzed for SIINFEKL-tetramer specificity by flow cytometry. Shown are representative FACS plots of each group of mice (n=3/group). To demonstrate tetramer specificity, FACS plots from intrahepatic Ova specific CD8+ T cells from an OT-I mouse were implemented subsequently (as indicated by #). These cells were not acquired at the same day as the experimental groups.

3.3 Providing antigen specific T cells by allogenic transplantations

As shown in chapter 3.2, the Ova x Cre model is immune competent, but does not allow the development of CD8+ T cell mediated immune responses against Ova which originate from endogenous T cell clones. To investigate the onset, course and fate of intrahepatic T cell responses, Ova specific CD8+ T cells (OT-I) derived from transgenic allogenic donor mice were transplanted into Ova x Cre recipient mice in the following experiments.

3.3.1 Adoptive transfer of Ova specific CD8+ T cells induces acute hepatitis

Neither induction of intrahepatic antigen expression alone, nor simultaneous induction of pro-inflammatory conditions could induce a CD8+ T cell mediated immune response against Ova. To evaluate whether a CD8+ mediated immune response towards peripheral antigen within the tolerogenic milieu of the liver could be achieved, allogenic transplantations of Ova specific CD8+ cells (OT-I) were performed. Upon adoptive transfer of 5 x 10^6 OT-I cells, kinetics of immune response were evaluated by measurement of serum ALT. Measurement prior to adoptive transfer confirmed that all recipients were healthy (Fig. 9). On day 1 upon adoptive transfer a slight increase of ALT was observed in Ova x Cre mice. This increase peaked at day 3 upon adoptive transfer in both, high and low antigen expressing Ova x Cre mice. The ALT declined to physiological levels by day 6 in both groups. The control groups remained healthy throughout the whole experiment (Fig. 9). Unexpectedly, the tissue damage was reciprocal to the amount of antigen presented, as the ALT was higher, the less the antigen was presented (Fig. 9).

In summary, these results indicate a specific CD8+ mediated immune response within the liver towards hepatic antigen expressed at high or low frequency.

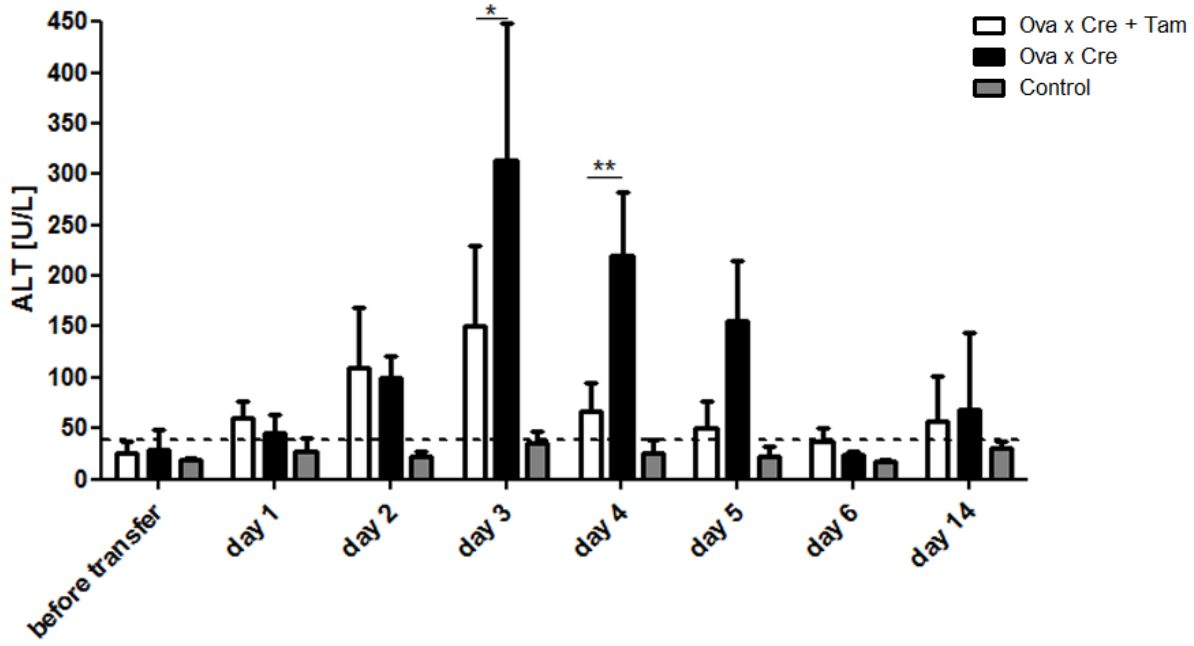

Fig. 9: Adoptive transfer of antigen specific CD8+ T cells induces acute hepatitis. Induction of hepatitis as indicated by ALT in the peripheral blood was measured on different time points upon adoptive transfer of 5*106 OT-I cells to high or low antigen expressing mice. Ova single transgenic mice were also treated with or without Tam and adoptively transferred to exclude unspecific side effects due to the treatment. Statistical analysis was performed using Mann-Whitney test with n≥4; *p≤0,0319; **p≤0,0095. The dashed line indicates the threshold of physiological ALT <40 U/L.

3.3.2 Clearance of low, but not high antigen is achieved within 13 days

Adoptively transferred OT-I cells seem to induce a liver damage towards both, high and low antigen levels in the liver as determined in chapter 3.3.1 (Fig. 9). This raised the question if the acute response and the accompanied liver damage on day 3 would lead to a reduction of antigen expressing hepatocytes. A reduction of cell numbers and thus indirectly of antigen load would be reflected by a decrease of *ova* sense mRNA. Thus, liver tissues of Ova x Cre and Ova single transgenic control mice were isolated on day 3 upon adoptive transfer of OT-I cells and the antigen expression was analyzed by qRT-PCR (compare Fig. 3A, Primer combination 1a/4a). As shown previously (Cebula *et al.*, 2013), both, high and low antigen levels remained unaltered if compared to non-transferred Ova x Cre mice (Fig. 10).

In summary, although an intrahepatic immune response is established upon adoptive transfer of OT-I cells, antigen expressing hepatocytes are not reduced at early time points.

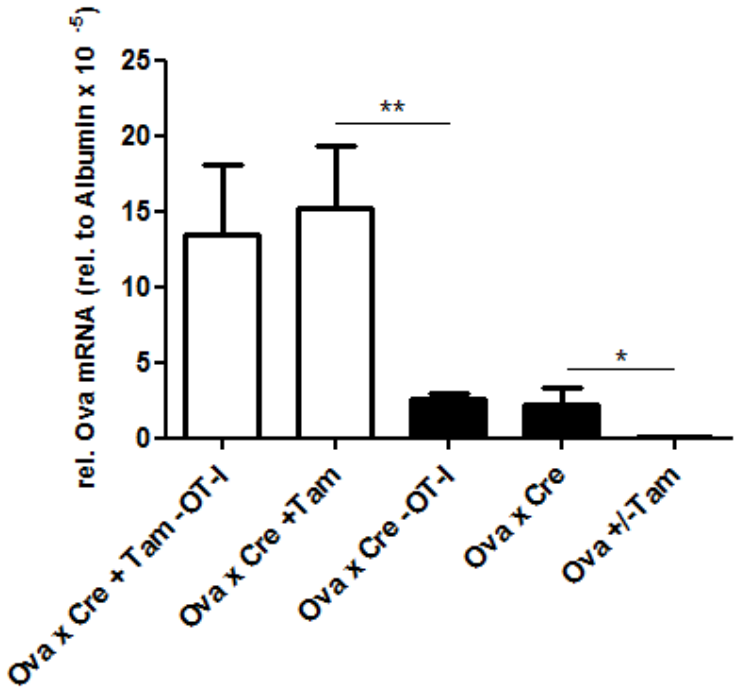

Fig. 10: Antigen burden remains unaltered during acute hepatitis. 3 days upon adoptive transfer of 5 x 10^6 naïve OT-I cells, recipient mice were sacrificed and liver samples were taken for qRT-PCR to determine the amount of antigen expression as described above. Despite acute liver damage, analysis no clearance or reduction of antigen load within this time period. n≥3, statistical analysis was performed using Mann-Whitney test. *p≤0,0176; **p≤0,0061.

Adoptive transfer of OT-I cells to high and low antigen expressing mice was shown to result in acute hepatitis accompanied by liver damage (Fig. 9). Yet, determination of antigen expressing levels at early stages of immunity has shown that the quantity of intrahepatic antigen remained unaltered (Fig. 10). This raised the question if the T cells required a longer time period to cure the antigen. To this end, 5 x 10^6 naïve OT-I cells were adoptively transferred to Tam treated and untreated Ova x Cre mice with high or low numbers of antigen expressing cells, respectively. Liver tissues were taken from recipient mice on day 13 to determine the amount of antigen expression by qRT-PCR for the sense orientation of *ova* mRNA. Analysis revealed clearance of antigen on day 13 as indicated by complete elimination of *ova* sense expression in low antigen expressing mice (Fig. 11). In contrast, *ova* mRNA within high antigen expressing mice was still high and remained unaltered if compared to mice that did not receive adoptive transfers of OT-I cells (Fig. 11).

Of note, clearance of low antigen amounts was detected as early as day 7 upon adoptive transfers, i.e. a time point when liver damage had already declined (data not shown). Yet, the

evaluation of different recipient mice revealed heterogeneous results, as some mice showed a clearance, whereas antigen was still expressed in other mice of the same group (data not shown). This indicates that clearance of low antigen started during the transient response but the kinetics of clearance varied among individual mice.

Taken together, the capability of CD8+ T cells to clear low but not high antigen amounts within the liver suggest a regulative mechanism of the antigen itself towards the immune response.

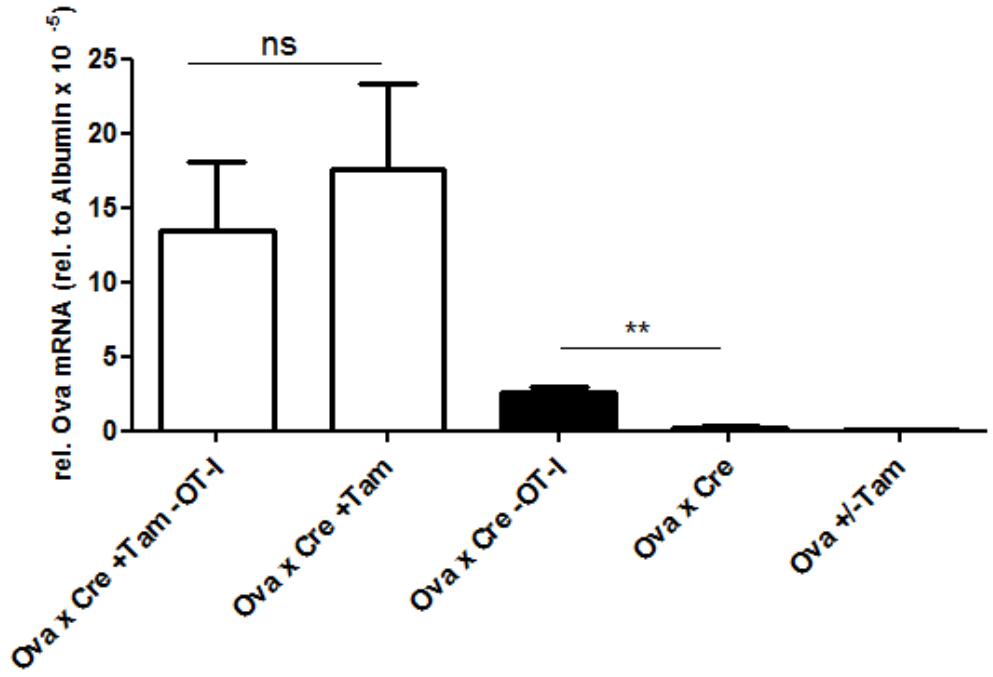

Fig. 11: Clearance of low antigen is achieved within 13 days upon adoptive transfer of naive OT-I cells. 13 days upon adoptive transfer of 5 x 10^6 naïve OT-I cells, recipient mice were sacrificed and liver samples were taken for qRT-PCR to determine the amount of antigen expression using the primer combination 1a/4a. Low amount of antigen becomes cleared, if compared to non-transferred Ova x Cre mice. Mean of n> 4 mice/ group ±SD; p= 0,0012; ns= non-significant; Mann-Whitney test. Expression values of non- transferred Ova x Cre mice (-OT-I) correspond to data as shown in Fig. 4A .

3.3.3 High intrahepatic antigen loads impair acute clearance

The data above indicate a failure of adoptively transferred T cells to clear high intrahepatic antigen loads. This raised the question whether the failure is due to the fact that the provided T cells were naive. To test this hypothesis, 5 x 10^7 OT-I cells were pre-activated *in vitro* in the presence of 3 µg/ml Ova peptide (Kb/SIINFEKL$_{257-264}$) 2 days prior adoptive transfer to induce an effector phenotype of T cells (T_{eff}). The activated phenotype of T cells was analyzed by flow cytometry 2 days upon peptide stimulation. Analysis of T cell frequencies by flow cytometry showed a ~5.2fold increase of CD8+ cells, if compared to unstimulated cells (compare Fig. 12, upper vs. lower panel). This suggests an antigen specific expansion of cells. To evaluate the status of cells in more detail, cells were stained for markers of activation. Indeed, cells that were cultured in the presence of SIINFEKL-peptide for 2 days showed expression of CD69, CD44 and PD-1 (Fig. 12, upper panel). In contrast, unstimulated cells showed only low upregulation of these markers, confirming an antigen dependent activation of CD8+ OT-I cells (Fig. 12, lower panel).

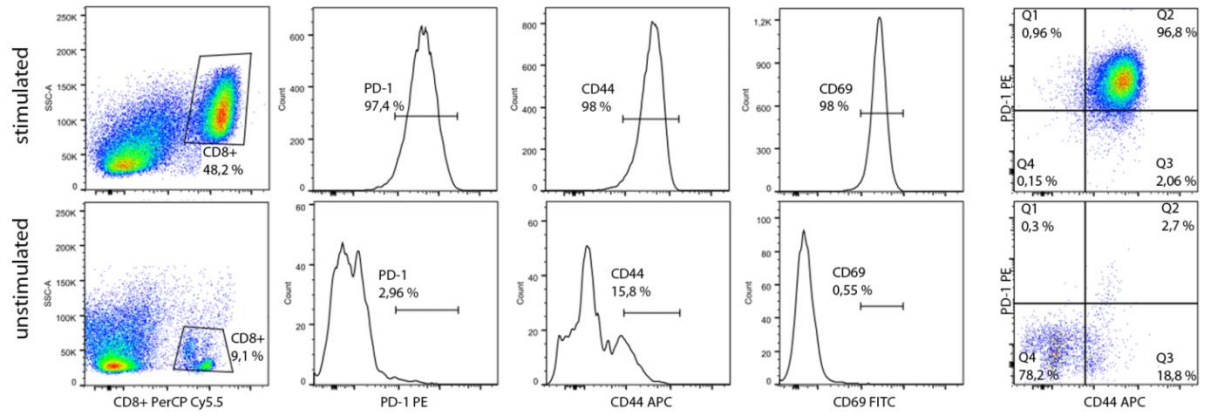

Fig. 12: Phenotypic characterization of *in vitro* pre-activated OT-I cells. OT-I cells were cultivated *in vitro* in the presence of 90 µg Kb/Ova257-264 peptide for 2 days (upper panel) or left unstimulated (lower panel). Cellular expansion and expression of activation markers CD69, CD44, PD-1 was performed by flow cytometry.

As the peptide dependent expansion and activation of CD8+ OT-I cells were confirmed, 5 x 10^6 pre-activated cells were adoptively transferred to Ova x Cre mice expressing high or low amounts of antigen within the liver, respectively. Ova single transgenic mice were treated with Tam or left untreated and served as controls to exclude immune mediated side effects in response to the activated status of T cells. ALT was measured on day 3 upon adoptive transfer to evaluate an onset of immune response. If compared to mice that received naïve OT-I cells, only a moderate liver damage in both, high and low antigen expressing recipients

was observed. Mice displayed slightly elevated, yet not dramatically increased ALT values of 60 to 75 U/L (data not shown). qRT-PCR on liver tissue isolated on day 13 showed that the high antigen load was not affected by adoptive transfer of T_{eff} cells, whereas the low antigen load was completely eliminated (Fig. 13A). This led to the conclusion that even responses of T_{eff} cells may become impaired by high levels of antigen presented within the liver. Of note, adoptive transfer of antigen specific CD8+ T cells to recipients expressing high levels of antigen led to significantly higher expression levels if compared to mice without transplantation of T cells (Fig. 13A). This might be due to the regenerative capacities of the liver (Michalopoulos & DeFrances, 1997; Michalopoulos, 2013). Since adoptively transferred OT-I cells induce an initial immune response towards high antigen presenting hepatocytes which is accompanied by liver damage, remaining hepatocytes in the ON state might contribute to the regeneration of the liver.

Adoptive transfer of naïve OT-I cells has shown that 5 x 10^6 cells are capable to clear the low antigen load (Fig. 11). As the antigen amount in Tam treated Ova x Cre mice is 5fold higher, it was speculated that higher numbers of OT-I cells could clear the induced antigen level. To investigate this hypothesis, 5fold more naïve OT-I cells (i.e. 2,5 x 10^7 cells) were administered to Ova x Cre mice expressing high intrahepatic antigen. The experiment was done over a time span of 5 weeks with five sequential transfers of 5 x 10^6 naïve OT-I cells, each. This setting was chosen to avoid adverse effects caused by injection of high numbers of OT-I cells at once. Low antigen expressing Ova x Cre mice which showed a clearance of antigen upon a single adoptive transfer, served as controls to exclude induction of bystander hepatitis due to T cell transfer as described in another setting (Beland *et al.*, 2012).

In agreement with previous experiments (Fig. 9), ALT levels showed a high liver damage on day 3 upon the first adoptive transfer of 5 x 10^6 OT-I cells to low antigen expressing mice, whereas a moderate liver damage was observed in high antigen expressing mice (data not shown). Upon the second adoptive transfer, low antigen expressing mice displayed physiological levels of ALT, indicating the absence of ongoing liver damage and thus excluding unspecific effects due to T cell transfer as reported previously by Beland and colleagues (Beland *et al.*, 2012). Repeated transfers of naïve OT-I cells to high antigen expressing Ova x Cre mice in contrast revealed moderate liver damage upon each adoptive transfer as indicated by ALT values of ~100 U/L. These ALT levels remained elevated but did not increase over time (data not shown). ALT reached physiological levels on day 16 upon the 5^{th} adoptive transfer. Mice were sacrificed and liver tissue was isolated to analyze the amount of antigen expression by qRT-PCR. High antigen levels were reduced upon transfer of 2,5 x 10^7 cells, if compared to treatment with 5 x 10^6 cells (Fig. 13B). However, a

significant number of antigen expressing cells remained and thus the high antigen could not be cleared completely as compared to low antigen expressing mice.

In summary, the results indicate that neither improved quality of T cells, nor increased quantity of T cells can finally eliminate high numbers of antigen presenting hepatocytes.

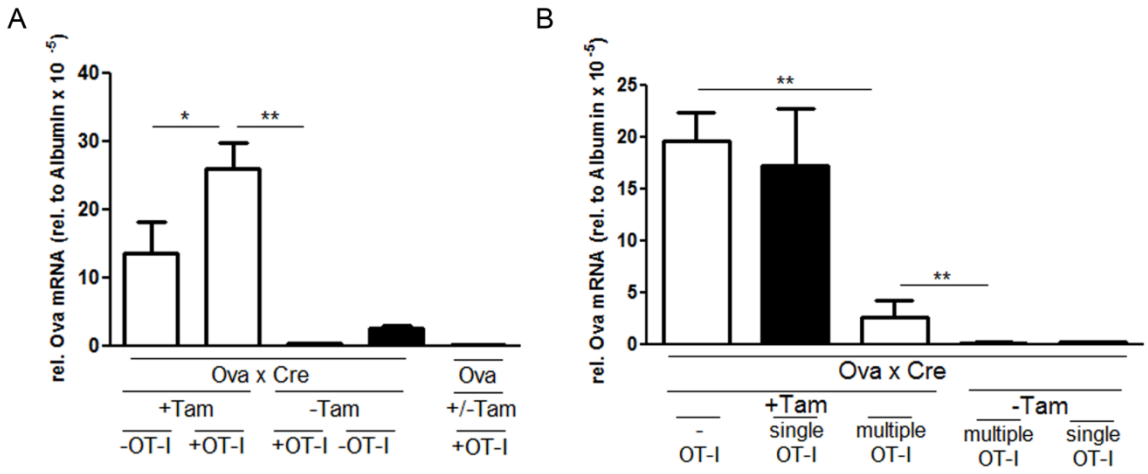

Fig. 13: High antigen load is not cleared by CD8+ T cells. 2 days prior to adoptive transfer OT-I cells were cultivated in vitro in the presence of Kb/Ova257-264 peptide for pre-activation of the cells. 5 x 10^6 OT-I cells were adoptively transferred to Ova x Cre mice expressing high or low antigen within the liver, Ova single transgenic mice served as controls. (A) On day 13 upon adoptive transfer liver tissue was isolated for qRT-PCR to determine the antigen expression level as described above. n≥4; **p≤0,01, Mann-Whitney test. (B) To rule out adverse target vs. effector ratios upon adoptive transfers of naïve cells, the quantity of provided OT-I cells was increased 5fold. In total 2,5 x 10^7 naïve OT-I cells were adoptively transferred to high and low antigen expressing mice. To avoid side effects by providing high numbers of T cells at once, 5 x 10^6 OT-I cells were adoptively transferred per week. n≥4; **p≤0,01; Mann-Whitney test. Antigen expression values in (A) of non-transferred Ova x Cre mice (-OT-I) correspond to the data as previously shown in Fig. 4A.

3.3.4 Antigen specific T cells are maintained upon clearance of antigen presenting hepatocytes

Adoptive transfer to both, high and low antigen expressing Ova x Cre mice, results in early activation and antigen specific proliferation of OT-I cells in liver, spleen and liver draining lymph nodes on day 3 upon adoptive transfer (Cebula *et al.*, 2013). Despite initial liver damage a clearance of antigen was only achieved by cells exposed to low antigen levels. High amounts of antigen, in contrast, remained unaffected if a single dose of naïve OT-I cells was provided (compare chapter 3.3.2 Clearance of low, but not high antigen is achieved within 13 days). This raised the question about the fate of OT-I cells exposed to different numbers of antigen as presented in hepatocytes of Ova x Cre mice. Thus, total leucocytes and OT-I cells in liver and spleen were calculated on day 13 upon adoptive transfer.

Calculation of total lymphocytes in the liver showed a significantly increased number of non-parenchymal cells (NPC) in low antigen expressing Ova x Cre mice if compared to high antigen expressing mice or controls (Fig. 14A). The determination of splenic lymphocytes showed no significant differences among the experimental groups (Fig. 14B). To determine the amount of antigen specific OT-I cells, calculation of total cell numbers was performed by staining liver and spleen resident cells for the congenic marker Thy1.1 (Fig. 14C and D). OT-I cell numbers were significantly increased in the livers of low antigen Ova x Cre mice if compared to mice expressing high amounts of antigen. Numbers of OT-I cells in high antigen expressing mice were as high as numbers in control mice, in which no activation of T cells was observed (Fig. 14C). As the transferred cells were isolated from spleens of donor mice, it was assumed that the cells relocated to the spleen of recipient mice if not activated. Indeed, evaluation of splenic resident OT-I cells showed that the highest number was found in the control mice (Fig. 14D). A slightly decreased number of OT-I cells resided within the spleen of low antigen expressing Ova x Cre mice. In contrast numbers of OT-I cells in high antigen expressing mice were significantly decreased (Fig. 14D).

Taken together, the results suggest maintenance of antigen specific CD8+ cells upon clearance of low antigen. T cells remain at the site of primary antigen exposure (i.e. liver) and secondary lymphatic tissue (i.e. spleen) of low antigen expressing mice. If T cells remain naïve due to absence of stimulating antigen, they seem to home to their initial lymphoid organ, the spleen, as seen in control mice, but become depleted if exposed to high antigen levels within the liver.

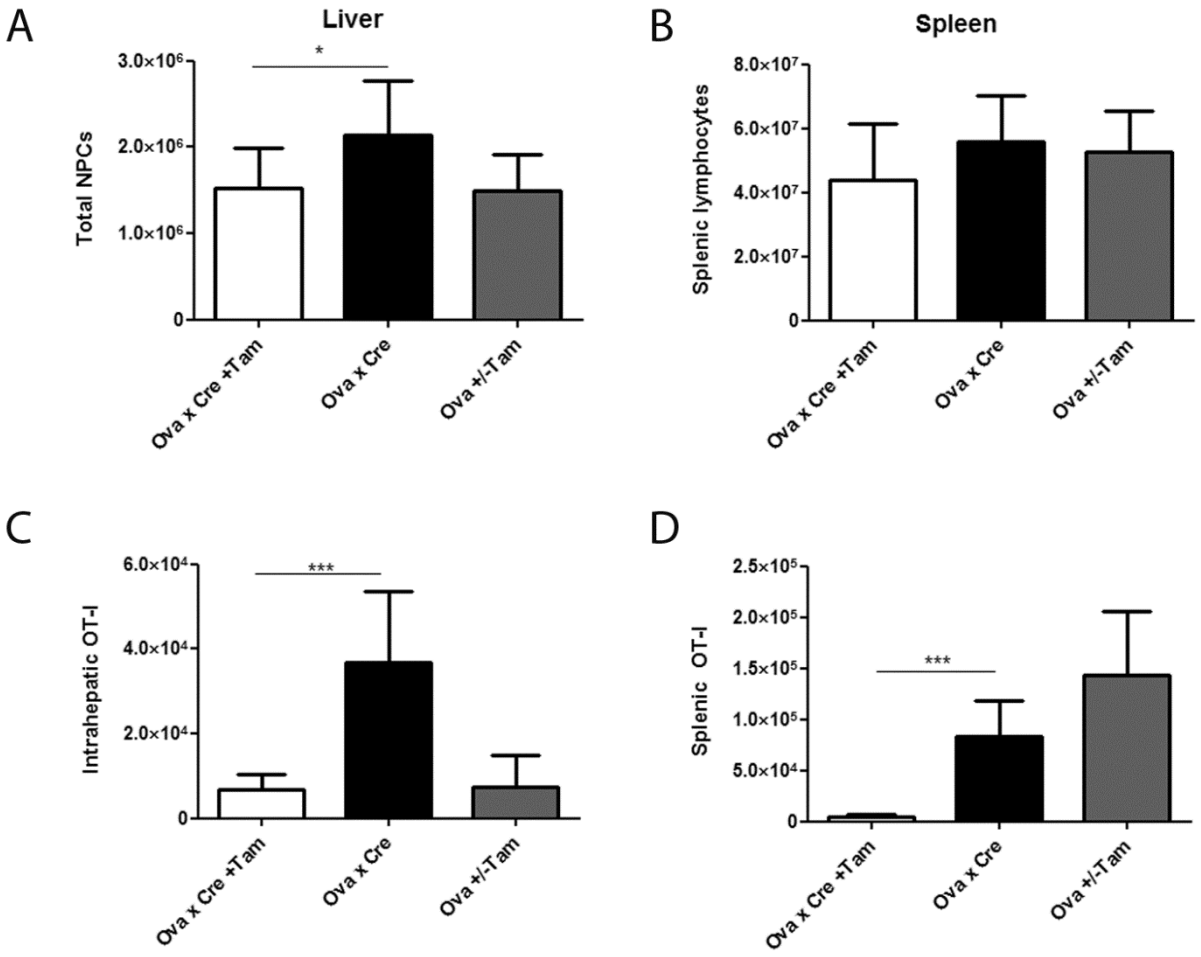

Fig. 14: Intrahepatic and splenic located OT-I cells are more abundant in low antigen expressing mice. Tam treated and non treated Ova x Cre and control mice were sacrificed on day 13 upon adoptive transfer of 5 x 10^6 naïve OT-I cells. Livers and spleens of recipient mice were isolated and tissue resident lymphocytes were purified to determine total numbers of leucocytes (A and B) or Thy1.1+ OT-I cells (C and D). Shown is the mean of n≥5 mice/group; *p≤0,05; **p≤0,01, using Mann-Whitney test.

3.3.5 Exposure to high antigen load induces T cell exhaustion

The marker CD69 was previously demonstrated to be expressed immediately during T cell activation as a *de novo* synthesized molecule (Castellanos Mdel *et al.*, 2002; Cebrian *et al.*, 1988). To investigate the presence of an activated phenotype of OT-I cells exposed to different antigen loads, OT-I cells were isolated on day 13 upon adoptive transfer and stained for CD69. CD69 was significantly upregulated on liver localized OT-I cells exposed to high antigen load as shown by the geometric mean fluorescence intensity (geom. MFI). In contrast, CD69 was only slightly upregulated on OT-I cells exposed to low antigen levels, whereas its expression was still lower on control cells (Fig. 15A). Evaluation of CD69 on spleen resident OT-I cells however did not show differences among the experimental groups (Fig. 15D). Since only low numbers of OT-I cells were present in high antigen expressing

mice they could have been deleted, as a consequence of T cell exhaustion (Wherry *et al.*, 2003). To investigate if the remaining cells displayed an exhausted phenotype, liver and spleen resident OT-I cells were stained for markers of exhaustion on day 13 upon T cell transfer. The markers PD-1 and Lag 3 have previously been reported to be expressed on exhausted T cells (Jackson *et al.*, 2013; Paley *et al.*, 2012). Liver resident OT-I cells showed a significantly higher expression of PD-1 if exposed to high antigen levels. In contrast, the expression of PD-1 on OT-I cells exposed to low levels of antigen was not increased if compared to control cells (Fig. 15B). Comparing the expression of Lag 3 on control cells to cells from low antigen expressing mice, a significant increase was observed in the latter group. However, Lag 3 was even higher expressed on liver resident OT-I cells derived from high antigen expressors (Fig. 15C). This increased expression of both exhaustion markers on OT-I cells exposed to high levels of intrahepatic antigen suggests an induction of T cell exhaustion in response to high numbers of antigen presenting hepatocytes.

Analysis of splenic OT-I cells revealed no differences in the expression of CD69 and PD-1 among the groups (Fig. 15D-E). Yet, differences were observed by analysis of the exhaustion marker Lag 3, which reflects the same results that were made on liver localized OT-I cells (Fig. 15E). However, the geometric MFI of Lag 3 on splenic OT-I cells was found to be 10fold lower if compared to liver resident OT-I cells (compare Fig. 15C to F).

In summary, the results indicate an activation of antigen specific T cells in both, high and low antigen expressors. Yet, the kinetics of activation appear to be different, as CD69 expression is significantly higher on OT-I cells exposed to high antigen amount, which might be due to sustained antigen expression. Moreover, high antigen amount seems to induce exhaustion of remaining antigen specific T cells in high antigen expressing mice, as determined by PD-1 and Lag 3.

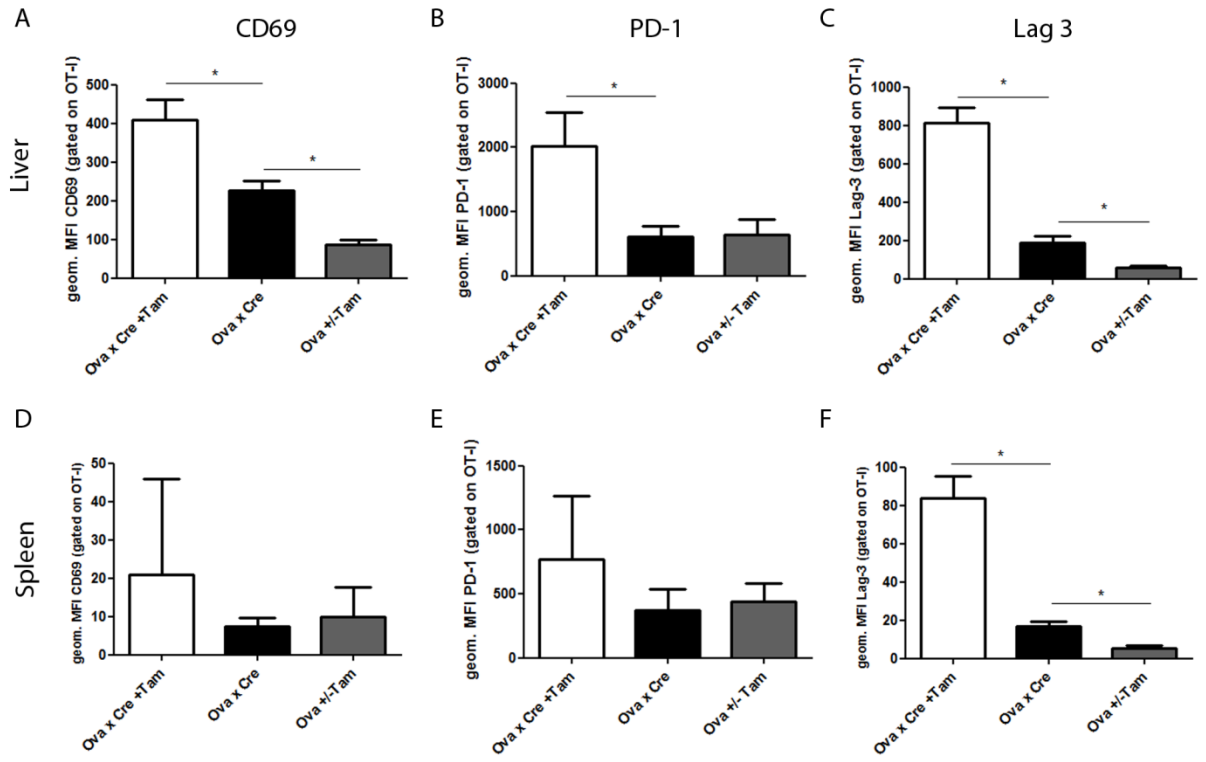

Fig. 15: High amounts of antigen induce exhaustion of antigen specific CD8+ T cells. Antigen specific Thy1.1+ OT-I T cells in liver (A-C) and spleen (D-F) were phenotypically characterized by flow cytometry on day 13 upon adoptive transfer of 5 x 10^6 OT-I T cells. Cells were stained for the activation marker CD69 (A and D) and the exhaustion markers PD-1 (B and E) or Lag 3 (C and F). Marker expression is depicted as geom. MFI. n≥3; *p≤0,05; Mann-Whitney test.

3.3.6 T cells exposed to low levels of antigen produce effector cytokines upon re-stimulation with target antigen

Upon antigen dependent activation, T cells gain the potential to produce and release effector cytokines such as IFNy or TNFα (Mueller *et al.*, 2013; Obar *et al.*, 2011; Seder *et al.*, 2008). To investigate if OT-I cells primed by different amounts of intrahepatic antigen were positive for these effector cytokines, intrahepatic and splenic OT-I cells were characterized for their functionality on day 13 upon adoptive transfer. *In vitro* cultivation of liver derived CD8+ T cells in the presence of Kb/Ova257-264 peptide induced no cytokine production if OT-I cells were exposed to high intrahepatic antigen load (Fig. 16). The cytokine profile of OT-I cells (Fig. 16A open graphs) was comparable to unspecific CD8+ T cells (Fig. 16A filled graphs). In contrast, strong expression of both, IFNy and TNFα was observed upon peptide stimulation of cells that were exposed to low antigen amounts within the liver (Fig. 16A). Moreover, these cells were double positive for IFNy and TNFa (Fig. 16). A similar pattern of antigen induced cytokine production could be detected in OT-I cells re-isolated from control mice (Fig. 16). By quantification of the respective cytokine production a significantly impaired

functionality of OT-I cells exposed to high antigen load became evident, if compared to cells exposed to low antigen amounts (Fig. 16B). Similar observations were made upon functional investigation of splenic derived OT-I cells (data not shown). Of note, unspecific stimulation with PMA/Ionomycin that activates the T cells in a TCR independent manner did not show differences between OT-I cells derived from high or low antigen expressing mice, respectively (data not shown).

Taken together, the results indicate a protective phenotype of antigen specific CD8+ T cells that were primed under low antigen conditions, whereas T cells exposed to high antigen burden seem to be exhausted in an antigen dependent manner.

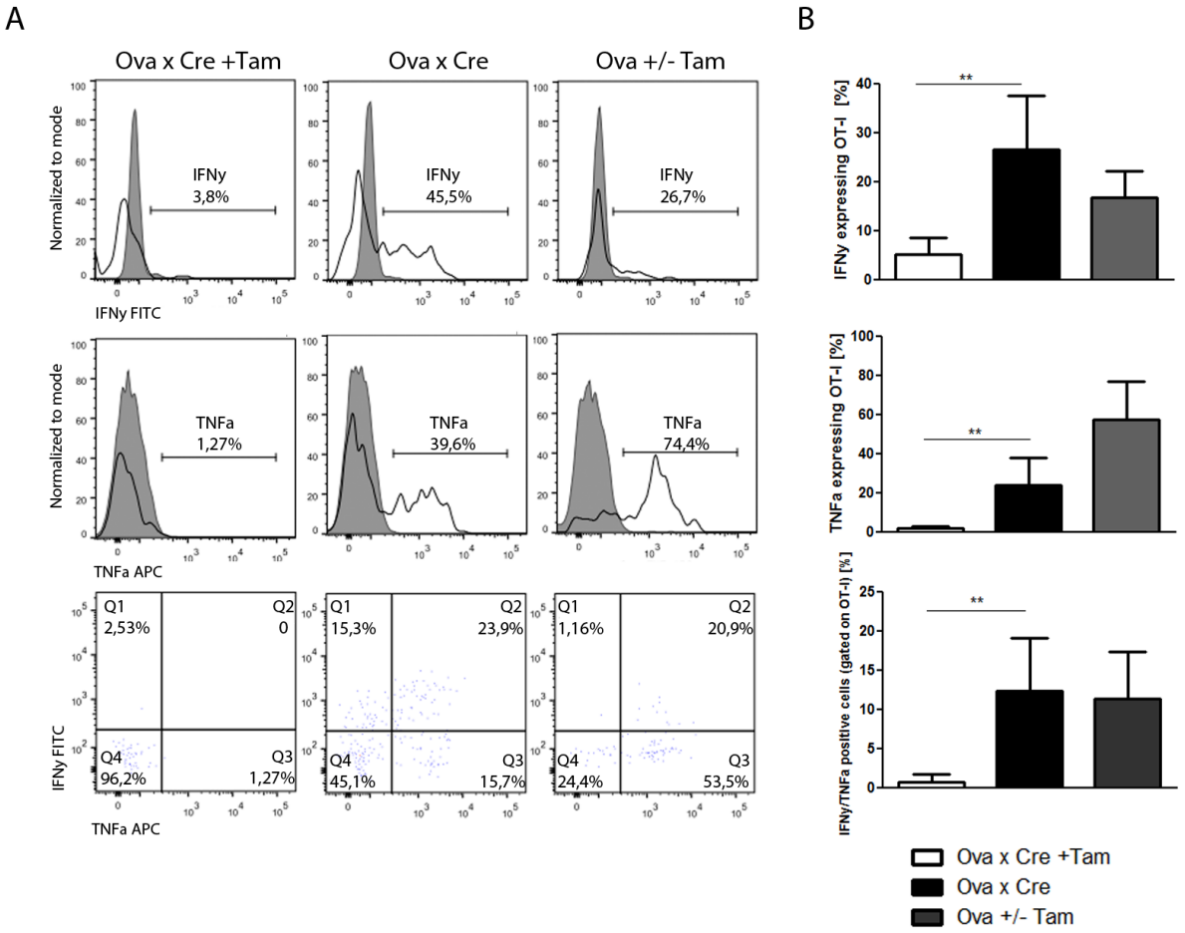

Fig. 16: OT-I cells exposed to low antigen levels produce effector cytokines. To evaluate effector function of intrahepatic primed Ova specific CD8+ T cells, OT-I cells were isolated on day 13 upon adoptive transfer and stimulated *in vitro* by 2,5 µg $Ova_{SIINFEKL}$ peptide/ ml to induce effector cytokine expression. 2 h upon stimulation vesicle trafficking was blocked by addition of Brefeldin A and cells were incubated for additional 5 h in the presence of Ova peptide. (A) To analyze production of effector cytokines in response to Ova, cells were stained for IFNy (upper panel) and TNFα (middle panel) and analyzed by flow cytometry. The respective cytokine expression of OT-I cells (open graphs) was compared to unspecific CD8+ T cells (filled graphs) which were considered to be negative in response to Ova. In addition to single production of effector cytokines, cells were also investigated for poly production of cytokines (bottom panel). (B) The quantification of effector cytokine production as shown in (A) from n≥5 mice/group. **p≤0,01 using Mann-Whitney test.

3.3.7 A protective immunity is established upon antigen clearance

The maintenance, activated phenotype and capability to produce effector cytokines of OT-I cells exposed to low levels of intrahepatic antigen suggests the establishment of a T cell memory (T_{mem}) population (Mueller *et al.*, 2013). T_{mem} develop upon successful clearance of an antigen and respond more rapidly to the same antigen than naïve cells do. Thus, T_{mem} provide a lifelong protection of the host´s integrity (Ahmed & Gray, 1996; Kaech & Ahmed, 2001). To investigate if a protective immune response was established upon clearance of low intrahepatic antigen, Ova x Cre mice were challenged by an *in vivo* cytotoxic T lymphocyte

(CTL) assay (Aichele *et al.*, 1997). *In vivo* CTL is a well-accepted method to investigate the presence of antigen specific T cells *in vivo*. To this end, splenocytes were isolated from unrelated BL/6 donor mice. One half of the splenocytes was pulsed with target peptide and reflected the antigen presenting target population. The other half of isolated splenocytes remained naïve and served as control population. Both populations were stained with an agent that allows tracing of cells (e.g. CFSE). The target population was stained stronger ($CFSE^{hi}$) than the control population ($CFSE^{lo}$), allowing to discriminate between both populations. Both populations were mixed in a ratio 1:1 and adoptively transferred to recipients that were expected to harbor antigen specific T cells that respond against the peptide presenting target population. Since the transferred cells were derived from spleens of donor mice, they were expected to home to the same organ within recipients and cause infiltration of antigen specific CTL, if present. As a consequence, the antigen specific CTL start to kill the target cells by specific lysis. Upon isolation of spleens the day after transfer the remaining CFSE stained cells can be purified and the decay of target cells in relation to control cells allows an estimation of antigen specific killing mediated by CTL.

To investigate the presence of T_{mem} specific for Ova upon clearance of low antigen, high or low levels of antigen were induced in Ova x Cre mice, respectively. Mice were adoptively transferred with 5 x 10^6 naïve OT-I cells and subsequently challenged by an *in vivo* CTL on either day 13 or day 27 upon adoptive transfer. Ova single transgenic mice in which OT-I cells were maintained, yet not responding to antigen were used as controls. An *in vivo* CTL on day 13 showed the potential of cytotoxic T cells to perform killing of $CFSE^{hi}$ labeled target cells in low antigen expressing mice (Fig. 17A). This killing efficiency was significantly increased, if compared to mice expressing high levels of antigen. The killing efficiency in high antigen expressing mice was comparable to the efficiency observed in the control mice (Fig. 17A). Similar observations were made, if the same setup was performed on day 27 upon adoptive transfer (Fig. 17B). However, if compared to day 13, the killing efficiency was ~3fold decreased (compare Fig. 17A to B).

Taken together, the results of the *in vivo* CTL suggest that the priming of antigen specific CD8+ T cells leads to the establishment of a T cell population that facilitates a rapid and functional response against reoccurring antigen.

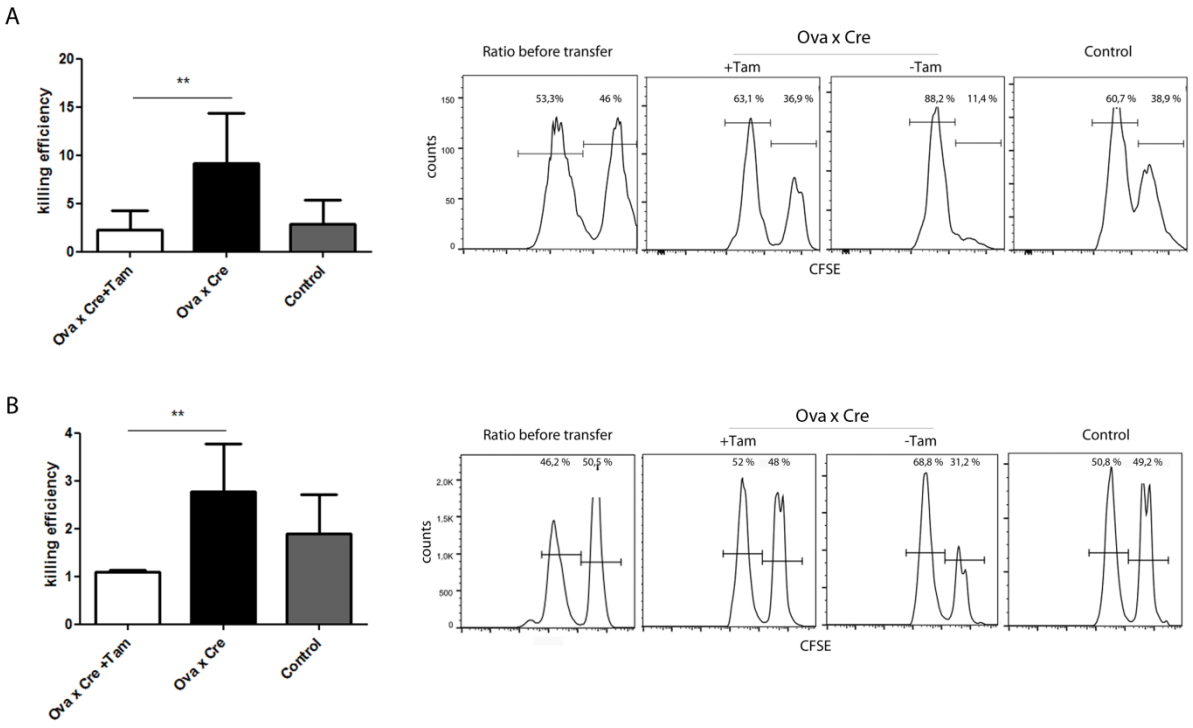

Fig. 17: Protective immunity in the periphery upon intrahepatic T cell priming. To investigate the presence of a potential memory T cell response upon intrahepatic priming of antigen specific CD8+ T cells, Ova x Cre mice were adoptively transferred with 5 x 10^6 naïve OT-I cells on day 0. $CFSE^{lo}$ labeled control cells and $CFSE^{hi}$ labeled Ova presenting target cells were adoptively transferred to recipient mice on day 13 (A) and 27 (B) upon adoptive transfer of OT-I cells. Single transgenic Ova mice were used as controls to investigate unspecific killing potential of naïve OT-I cells. 18 h upon transfer of CFSE labeled target cells, the spleens of recipient mice were isolated and analyzed by flow cytometry for the killing of target cells. The specific killing potential was represented by disappearance of $CFSE^{hi}$ cells. To calculate the specific killing efficiency, the ratio of $CFSE^{lo}$ vs. $CFSE^{hi}$ cells was determined prior transfer to recipient mice by flow cytometry. **p≤0,01 using Mann-Whitney test (n≥5 mice/ group).

3.3.8 Antigen experienced cells infiltrate the liver in response to high antigen

Priming of CD8+ T cells by low amount of intrahepatic antigen leads to antigen clearance and maintenance of protective CD8+ T cells. These observations led to the suggestion that CD8+ T cells primed by low amount of antigen were capable to clear high antigen amount, if re-challenged. To this end, OT-I cells were adoptively transferred to low antigen expressing mice on day -13. Subsequently, high amounts of antigen were induced by Tam application on day 0 (Fig. 18A). ALT analysis on day -10 confirmed induction of intrahepatic immune response to low levels of antigen (Fig. 18B). Upon induction of high antigen load in a subset of mice, ALT levels were measured again. Analysis of ALT on day 3 showed the absence of liver damage, whereas untreated Ova x Cre mice showed physiological levels of ALT (Fig. 18B). The absence of liver damage at this time point in high antigen expressing mice might either be due to a tightly controlled immune response or to induction of exhaustion by high antigen load. To test the latter, T cells from liver and spleen were isolated on day 13 and cell

counts of OT-I were determined. Quantification of liver derived OT-I cells showed a significantly increased number of cells in high antigen expressing Ova x Cre mice if compared to low expressors or control mice (Fig. 18C). Analysis of splenic derived OT-I cells showed low numbers of OT-I cells in spleens of high antigen expressing mice, whereas ~3-4fold increased amounts could be found in spleens of low antigen expressing mice or controls, respectively (Fig. 18C).

In summary, clearance of low antigen leads to establishment of a protective T cell population which is recruited into the liver upon subsequent challenge with high antigen levels. However, the antigen specific T cells fail to induce an ongoing immune response.

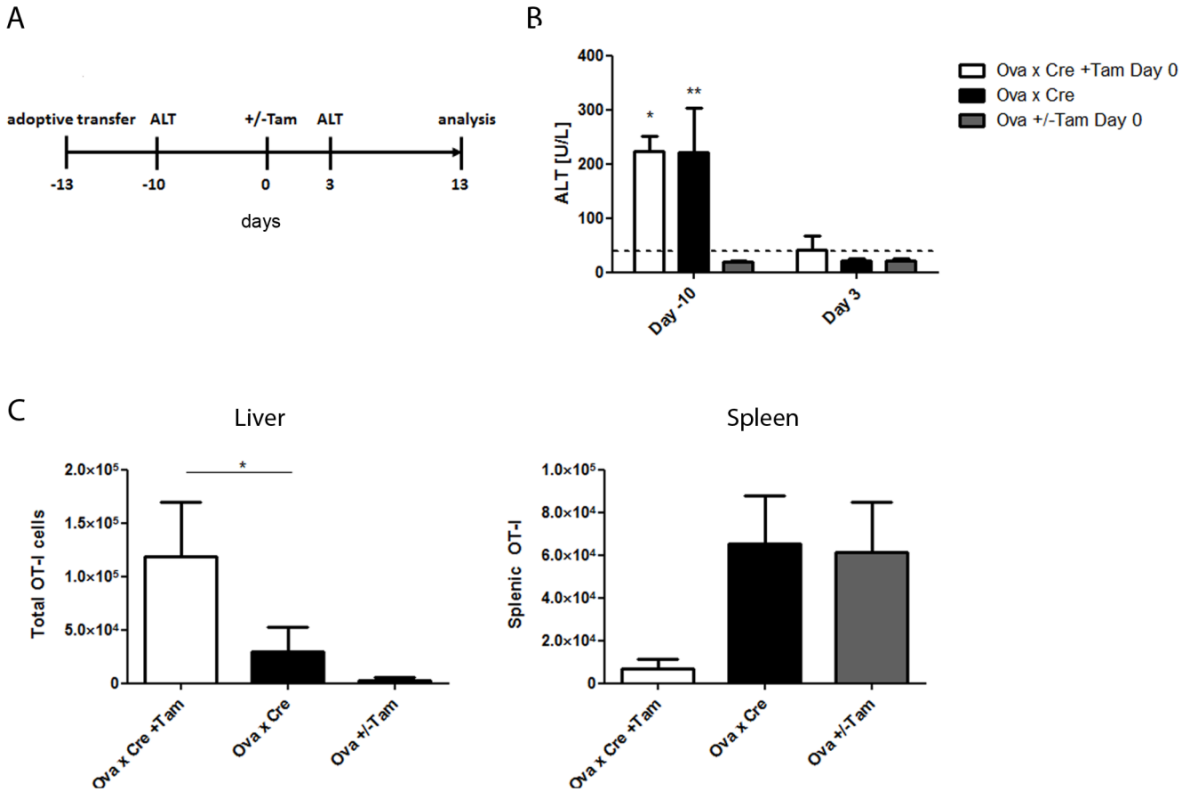

Fig. 18: T effector cells cannot overcome high intrahepatic antigen load. (A) Overview of the experimental setup to investigate T cell priming by low antigen conditions and subsequent challenge with high intrahepatic antigen load on the days indicated. (B) Evaluation of immune pathology within the liver as determined by serum ALT levels (±SD). Onset of liver pathology was determined by ALT values >40 U/L as indicated by the dashed line. (C) On day 13 upon induction of high intrahepatic antigen load cell numbers of OT-I cells from liver and spleen were determined (mean of n≥3 ±SD; *p≤0,05; Mann-Whitney test).

3.3.9 Subsequent challenge with high antigen load induces T cell exhaustion

The results shown above indicate an induction of T cell exhaustion that depends on the amount of antigen presented within the liver. To test this hypothesis, phenotypes of liver and spleen derived OT-I cells were analyzed by flow cytometry on day 13 upon induction of antigen load according to the experimental outline in Fig. 18A. Liver localized OT-I cells from both, low antigen expressing mice and low antigen expressing mice subsequently challenged with high antigen burden, showed a significantly increased expression of CD44 if compared to control mice (Fig. 19A). This indicates an activation of the cells due to the priming by low amounts of antigen. Evaluation of PD-1 expression showed that PD-1 was upregulated under both conditions, high and low antigen amount. The expression of PD-1 was found to be significantly different compared to OT-I cells isolated from control mice (Fig. 19B). However, the expression of this exhaustion marker was significantly increased on cells which were re-challenged with high intrahepatic antigen load if compared to low antigen expressing animals (Fig. 19). The same difference was observed by evaluation of PD-1 expression on spleen derived OT-I cells. Cells which were subsequently challenged by induction of high antigen load expressed a significantly higher amount of PD-1, while cells from low antigen expressing mice in this organ did not differ if compared to control mice (Fig. 19B).

Taken together, analysis of OT-I phenotypes upon subsequent challenge with high antigen load indicates an induction of T cell exhaustion towards antigen experienced cells which is dependent on the amount of antigen the cells were exposed to.

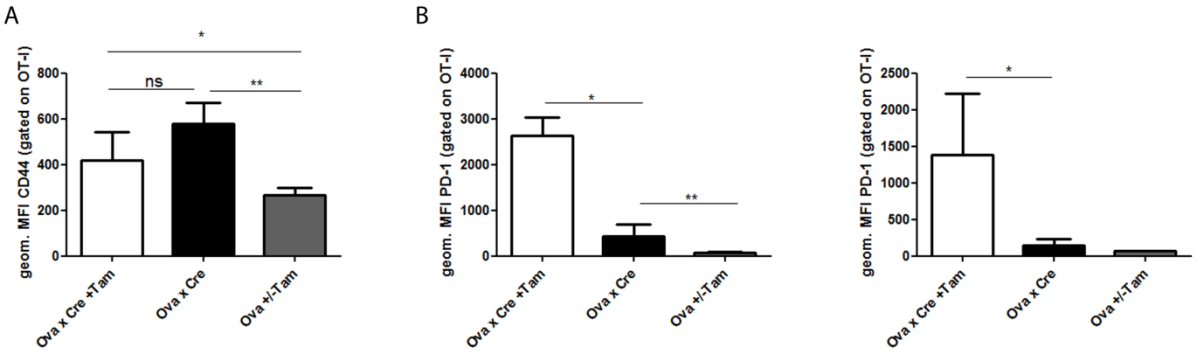

Fig. 19: Antigen experienced T cells become exhausted upon exposure to high numbers of antigen presenting cells. 5 x10^6 naïve OT-I were adoptively transferred to Ova x Cre mice, expressing low amounts of antigen on day -13. On day 0, a subset of mice was treated with Tam to subsequently challenge T_{eff} cells with high burden of intrahepatic antigen load. OT-I cells were isolated from liver and spleen on day 13 upon subsequent challenge with a high amount of intrahepatic antigen and analyzed for markers of activation (A) or exhaustion (B) by flow cytometry. Shown is the mean of n≥3 mice/group ±SD as depicted by the geom. MFI. *p≤0,05; **p≤0,01; ns= non-significant; Mann-Whitney test.

3.3.10 High antigen load within the liver cannot be overcome by antigen experienced T cells

Subsequent challenge of antigen specific CD8+ T cells with high amount of intrahepatic antigen shows enhanced T cell migration to the site where antigen is re-induced. Phenotypic characterization of T cells revealed significantly increased expression of the exhaustion marker PD-1 and no signs of liver pathology were detected. Yet, the T cell migration into the liver raised the question if the amount of antigen expressing cells was reduced by antigen specific T cells or whether it remained unaffected. To test, if this enhanced T cell migration has an impact on the antigen, the amount of intrahepatic antigen was determined within Ova x Cre mice that were treated with Tam upon clearance of low antigen levels. On day 13 upon induction of high antigen load, Tam treated and untreated Ova x Cre mice were sacrificed and liver tissue was isolated to perform qRT-PCR against the sense orientation of *ova* mRNA. The antigen was cleared in Ova x Cre mice that were not treated with Tam (Fig. 20). However, analysis of antigen expression within Ova x Cre mice in which high amounts of antigen were induced upon clearance of low levels showed that *ova* was expressed to high levels (Fig. 20). In accordance with the data above, these results indicate that indeed the amount of antigen negatively correlates to T cell function and thus determines intrahepatic immune responses.

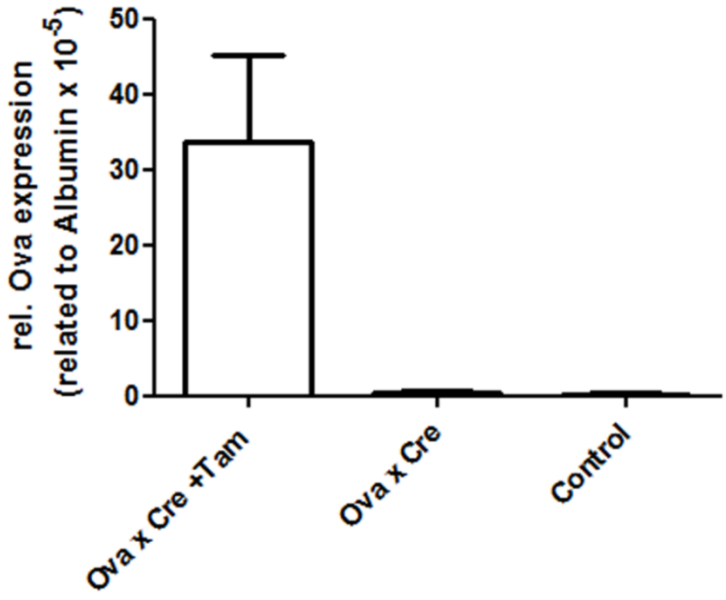

Fig. 20: High amount of antigen cannot be overcome by *in vivo* primed antigen specific CD8+ T cells. 5 x 10^6 naïve OT-I cells were adoptively transferred to Ova x Cre mice, expressing low levels of intrahepatic antigen on day -13. On day 0 when antigen was cleared, a subset of mice was with Tam to induce high antigen expression in

the liver. On day 13 RNA was isolated from liver tissue to evaluate the amount of intrahepatic antigen upon subsequent Tam treatment by qRT-PCR, (mean of n≥4 mice/group ±SD).

3.3.11 Functional failure of antigen experienced T cells exposed to high amounts of intrahepatic antigen

To confirm that T cell exhaustion depends on the amount of intrahepatic antigen, 5 x 10^6 naïve OT-I cells were adoptively transferred to Ova x Cre mice expressing low levels of intrahepatic antigen as described above (compare 3.3.9 Subsequent challenge with high antigen load induces T cell exhaustion). These conditions allow the generation of protective immunity by priming antigen specific CD8+ T cells *in vivo*. As described in chapter (3.3.9 Subsequent challenge with high antigen load induces T cell exhaustion) a subset of Ova x Cre mice was subsequently treated with Tam upon clearance of low antigen. *In vivo* primed antigen experienced OT-I cells were challenged by an *in vivo* CTL on day 14 upon subsequent induction of high antigen load (Fig. 21A). Flow cytometric analysis of target cell killing showed that Ova x Cre mice in which low amount of antigen was cleared (compare Fig. 20) displayed a high killing efficiency (Fig. 21B). In contrast OT-I cells, that were subsequently challenged by high antigen levels showed a killing efficiency that was dramatically reduced and comparable to the controls (Fig. 21B).

In summary, these results indicate that the number of antigen presenting hepatocytes governs the CD8+ mediated immune response. While low levels can be cleared and favor a potent memory population, high levels of antigen induce T cell exhaustion.

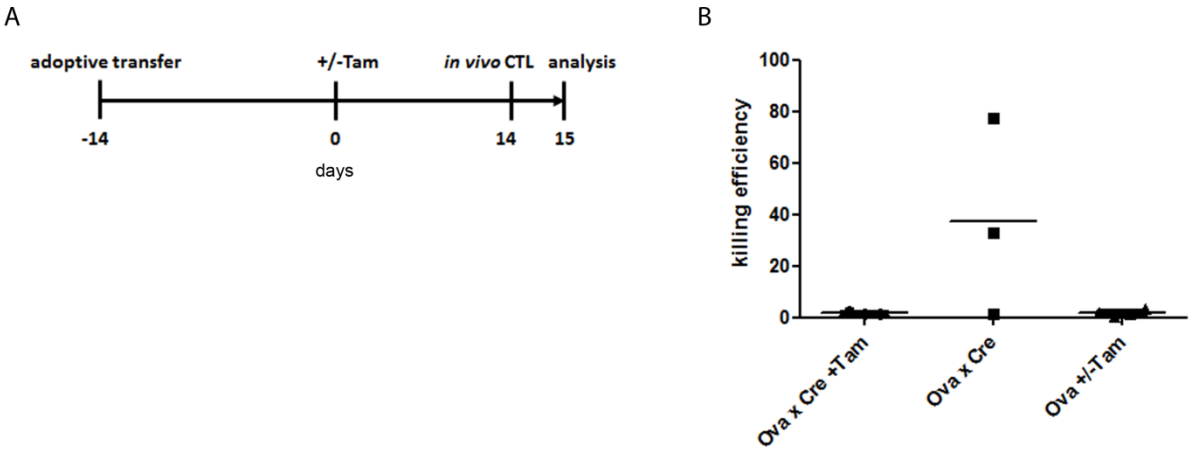

Fig. 21: High amount of intraheptic antigen impairs effector function of antigen experienced CD8+ T cells. 5x 10^6 naïve OT-I cells were adoptively transferred to Ova x Cre mice, expressing low levels of intrahepatic antigen as indicated in (A). OT-I were subsequently challenged by high amounts of intrahepatic antigen in a subset of mice and 14 days later an *in vivo* CTL was performed to investigate T_{eff} function. (A) Experimental procedure to investigate effector function of antigen experienced T cells exposed to high amounts of intraheptic antigen. (B) Analysis of specific target cell killing on day 15 upon subsequent induction of high intrahepatic antigen amount. $CFSE^{lo}$ control cells and $CFSE^{hi}$ labelled target (Ova pulsed) cells were adoptively transferred to recipient mice on day 14. To analyze the killing efficiency, spleens of recipient mice were isolated on the following day and analyzed for the presence of $CFSE^{hi}$ cells by flow cytomertry. Shown is the indicidual killing effeiciency from 3 mice/group.

4. Discussion

4.1 Onset and regulation of immune responses

Every organism is continuously exposed to microorganisms or mutagenic agents. However, severe diseases only occur rarely. The protection against these threats is mediated by the immune system that protects the host towards potentially harmful self or non-self factors. To ensure the protection of the host, the immune system has to be a very active organ with the aim to sense any kind of foreign factor that might reflect a possible threat to the host. Thus, the immune system consists of non-static cells which are specialized towards different functions (Parkin & Cohen, 2001). The immune system is divided into two major arms which are distinguished by speed and specificity of reaction: the innate and adaptive immunity. The innate immunity provides an antigen unspecific, but immediate response and is highly conserved among animals (Parkin & Cohen, 2001). In contrast, the adaptive immune response mediates antigen specific reactions which are tightly controlled but it requires several days or weeks to become established (Ahmed & Gray, 1996; Delves & Roitt, 2000a; Delves & Roitt, 2000b). Moreover, cells of the adaptive immune system, namely T- and B-lymphocytes, have the advantage to develop a memory which provides a vigorous and rapid response against a given epitope upon re-infection. Antigen specific B cells release antibodies and mediate the humoral response against extracellular pathogens, while cellular immunity is directed against pathogens that are characterized by an intracellular lifestyle and involves cytotoxic T cells. To exert immune responses, T cells require the presentation of pathogen derived peptides in the context of MHC class I or class II molecules, presented by the infected cell.

Especially CD8+ T cells are crucial mediators in the defense mechanism against viruses, including hepatotropic pathogens, namely, hepatitis B and hepatitis C virus (Protzer *et al.*, 2012; Thimme *et al.*, 2001; Thimme *et al.*, 2002). Yet, treatment of liver infections remains a major challenge for current medicine. Despite the development of a cellular antiviral immune response, ~70% of HCV infected patients display a failure of acute clearance and progress towards viral persistence. Therefore, determinants of viral clearance vs. chronic infection remain elusive. A major limitation to investigate antiviral immunity in hepatotropic infections is the species specificity of both, HBV and HCV, as these viruses strictly infect primates (Frentzen *et al.*, 2014; Huang *et al.*, 2012). These facts cause a restricted availability of laboratory animals, such as the mouse to reconstitute viral infection and antiviral immune responses.

To investigate the consequences of viral antigen expression, the transgenic mouse model Ova x Cre was designed to mimic the onset of neo-antigen expression exclusively in hepatocytes during liver infection within an immune competent mouse model. The model expresses the antigen Ova exclusively on hepatocytes upon Tam mediated induction of $CreER^{T2}$ which is integrated into the *albumin* locus (Sandhu *et al.*, 2011; Schuler *et al.*, 2004). The expression of Ova is mediated in a stochastic manner, giving rise to 50% of hepatocytes expressing the antigen (Fig. 3 and (Cebula *et al.*, 2013)). This model system was used within the present study to investigate the mechanisms of intrahepatic CD8+ T cell responses. Beside the analysis of T cell priming by non-professional antigen presenting cells and the induction of immune response, the influence of different antigen amounts within the liver was the specific focus of this work. The analysis revealed that adoptively transferred CD8+ T cells are primed within the liver and induce the destruction of antigen presenting liver target cells. Low amounts of antigen are cleared and lead to the establishment of memory cells, while high amounts of antigen induce T cell exhaustion.

4.2 Impairment of endogenous CD8+ T cell responses in the immune competent mouse model Ova x Cre

The transgenic mouse model Ova x Cre is a sterile model for infection, since antigen presentation is restricted to hepatocytes, requires application of Tam and does not depend on infection. Since the antigen is not secreted, only MHC class I restricted CD8+ T cells can be activated by induction of antigen expression.

Initial characterization of the Ova x Cre model has shown the development of tetramer specific CD8+ T cells upon vaccination. Yet, such antigen specific T cells are absent upon induction of antigen by Tam (Hillebrand and Wirth, unpublished). The fact that tetramer specific CD8+ T cells are generated upon vaccination implies the absence of a central tolerance mechanism within the thymus (Miller & Basten, 1996). Moreover, these data also exclude the presence of any peripheral tolerance which might be induced by APC´s in their housekeeping role upon uptake of Ova within the periphery (Parkin & Cohen, 2001). Indeed, deeper characterization of the Ova x Cre model revealed that the antigen is exclusively expressed by hepatocytes and moreover, is not secreted as expected from the design of the model. This becomes evident by the following observations:

- *in vitro* co-cultivation of OT-I cells with NPC from Tam treated and untreated Ova x Cre mice did not result in activation of OT-I cells (Sandhu *et al.*, 2011). If the

antigen was secreted, the NPC should have taken it up according to their role as cross-presenting cells (Ebrahimkhani *et al.*, 2011).

- Cross-breeding of Ova x Cre mice to mice bearing CD4+ T cells, which are specific for Ova (OT-II), did not result in the development of natural or induced T_{reg} (Fig. 5).
- numbers of peripheral OT-II cells were not found to be increased upon both, co-cultivation with hepatocytes derived from Ova x Cre mice (Hillebrand and Wirth, unpublished) and upon induction of antigen presentation in vivo (see chapter: 3.2.1 Central and peripheral tolerance are absent within Ova x Cre mice and data not shown).

These data imply the immune competence of the underlying Ova x Cre model which is characterized by the absence of thymic expression of the antigen. Together, the data confirm a specific expression of antigen only in hepatocytes and the absence of cross-presentation before initial liver damage by adoptively transferred OT-I cells. However, the results shown here do not exclude the induction of cross-presentation on MHC class II molecules upon liver damage by CD8+ T cells. To answer this question a double transfer of CD8+ and CD4+ Ova specific T cells would be required.

Moreover, it remains to be elucidated why the antigen expression per se does not result in clonal expansion of SIINFEKL-specific CD8+ T cells (Hillebrand and Wirth, unpublished). The absence of antigen specific CD8+ T cells upon induction of antigen reflects the situation as often observed in patients infected with HBV or HCV. In both cases an incubation period is observed during which high titers of virus are present within hepatocytes, but tolerated by the immune system (Rehermann, 2013). This tolerogenic effect is supposed to be established by the liver itself. Indeed, by ubiquitous expression of anti-inflammatory cytokines, such as IL-10 or TGFß, the liver establishes an immune suppressive environment and thus, prevents the onset of immune responses (Tiegs & Lohse, 2010). In addition to the suppressive cytokine milieu, liver resident cells of the NPC-fraction were also demonstrated to exert suppressive effects on the induction of CD8+ mediated immune responses. Especially the LSEC and HSC mediate cell to cell interaction with CD8+ T cells upon priming by hepatocytes and veto the previous activation of antigen specific CD8+ T cells, causing a tolerogenic phenotype (Limmer *et al.*, 2000; Schildberg *et al.*, 2008; Schildberg *et al.*, 2011).

Taken together, the underlying mouse model Ova x Cre mimics the situation of patients upon viral liver infection. The absence of central or peripheral tolerance indicates the immune competence of the model. This is confirmed by the establishment of Ova specific CD8+ T cells upon DNA vaccination. Together, these properties render the model to be a useful tool to explore intrahepatic immune responses towards neo antigens. The model allows both,

applications to overcome the tolerogenic liver milieu and reconstitution of CD8+ mediated T cell responses within the tolerogenic liver milieu.

4.3 Applications to overcome intrahepatic tolerance

Due to the absence of endogenous CD8+ T cell responses upon both, induction of Ova within the liver and DNA vaccination, it was speculated that the local environment of the liver exerts immune suppression. To overcome a potential intrahepatic peripheral tolerance that might impair the development of antigen specific T cells, in this work various pro-inflammatory conditions were established simultaneously to Ova expression. Yet, none of the provided pro-inflammatory conditions led to the development of an immune response directed against Ova. When antigen induction was accompanied by infection with adenoviral vectors or MCMV, the induction of intrahepatic immunity was induced as indicated by increased serum levels of ALT (Fig. 7). Liver localized CD8+ T cells from responding mice were increased in frequency, suggesting that these cells mediated the observed liver pathology. However, using a SIINFEKL-tetramer showed absence of Ova specific CD8+ T cells upon infection with either virus (Fig. 8 and data not shown). This suggests that the T cell response is primarily directed against adenoviral or MCVM specific antigens. The absence of Ova specific CD8+ T cells suggests that the Ova antigen was ignored by the immune system even under pro-inflammatory conditions. This ignorance might be due to a competition among immune cells for strong antigens. The establishment of such a competition in this work is quite probable. Although 50% of hepatocytes express Ova, the protein is only expressed at physiological levels by the *Rosa26* promoter (Nyabi *et al.*, 2009) while high amounts of virus were used which could cause the T cells to ignore the Ova. This difference of available antigens might shift the balance towards adenoviral directed immunity.

However, it was expected that the immune mediated liver damage in response to infection would also result in generation of Ova specific T cells. By uptake of cellular debris upon immune responses against adenoviral infected hepatocytes, during the ongoing immune response APC should also have cross-presented hepatocyte derived Ova peptide to specific CD8+ T cells localized in secondary lymphatic organs. An explanation for the observed lack of Ova specific CD8+ T cells might be the dynamics of immune response. Probably, the cross-presentation took place at the peak of antiviral response during which adeno specific CD8+ cells might outcompete Ova specific cells. To investigate if the use of adenovirus would break the suggested peripheral tolerance against Ova, an adenovirus expressing Ova protein could be of help. By this application, an immune response against adenoviral

epitopes would be established, but also against Ova as the amount of target peptide would be much higher than the *Rosa26* derived antigen levels. Additionally, cross-priming of Ova peptide could be realized by professional APC at the onset of immunity.

Interestingly, an intrahepatic immune response against hepatocyte derived antigen was only established when viruses were used to induce pro-inflammatory conditions. In contrast, triggering of TLR´s did not result in liver damage (3.2.4 Adenoviral infection induces CD8+ T cells, but does not lead to Ova specific T cell clones). It could be excluded that the TLR activation was not sufficient to induce inflammation: By use of a mouse expressing the reporter gene luciferase under the IFN-induced Mx-promotor (Pulverer *et al.*, 2010), intrahepatic TLR engagement was observed by increased luminescence within the liver (data not shown). The absence of intrahepatic immune response upon TLR engagement is unexpected, since it was previously demonstrated that PolyI:C induced engagement of TLR-3 recruits antigen specific CD8+ T cells ($LCMV_{gp33-41}$) in the liver and leads to tissue destruction as determined by increased serum ALT (Lang *et al.*, 2006). However, if compared to the present work, Lang *et al*. made use of adoptive transfer experiments to induce T cell infiltration, while TLR in this work were triggered to induce endogenous CD8+ T cells. Thus, the discrepancy might be explained by the different numbers of available T cells.

Taken together, despite the immune competence of the underlying mouse model, the induction of Ova expression exclusively in hepatocytes does not lead to the development of antigen specific CD8+ T cells derived from the endogenous pool. However, by the use of viruses to induce pro-inflammatory conditions the current work demonstrates the capability of the endogenous immune system, especially the CD8+ T cells, to mount an immune response against liver targeting pathogens. Other reports in contrast, provided antigen specific CD8+ T cells by adoptive transfers coinciding with infection (Lang *et al.*, 2006; Stabenow *et al.*, 2010). The present results indicate that the tolerogenic milieu of the liver can be overcome by the endogenous immune system. Yet, the tolerance of the immune system towards hepatocyte derived Ova could not be broken by the chosen settings. This failure might depend on a lack of supporting T_{helper} cells, as previously reported in a mouse model for liver inflammation (Derkow *et al.*, 2011). Unpublished data from our laboratory show the development of antigen specific CD8+ T cells upon plasmid DNA vaccination. These tetramer specific CD8+ T cells are localized within the liver and spleen of both, antigen expressing mice and controls. Interestingly, these cells do not overcome the high amount of antigen, but are not tolerated systemically, as a peripheral challenge of these cells with antigen pulsed target cells by an *in vivo* CTL shows specific killing (Cebula and Wirth, unpublished). These data indicate regulative capacities of the antigen itself in controlling the

outcome of intrahepatic immune response by either inducing T cell exhaustion or allowing clearance of antigen presenting hepatocytes.

4.4 Liver primed T cell responses

To study CD8+ T cell mediated immune responses towards hepatocyte derived antigen, in the second part of this thesis, antigen specific CD8+ T cells were adoptively transferred to Ova x Cre mice. It was of interest, if these cells were able to overcome the tolerogenic milieu of the liver and might establish a potent immune response in the absence of pro-inflammatory stimuli. If compared to other tissues, the unique vasculature of the liver allows a direct interaction between naïve T cells and antigens presented on MHC class I molecules by non-parenchymal cells and hepatocytes, leading to full priming of antigen specific CD8+ T cells (Bertolino *et al.*, 2001; Ebrahimkhani *et al.*, 2011; Warren *et al.*, 2006). In contrast to the classical hallmark of T cell activation (Bennett *et al.*, 1998; Ridge *et al.*, 1998; Schoenberger *et al.*, 1998), the activation of CD8+ T cells within the liver is even mediated in the absence of supporting CD4+ T cells (Bertolino *et al.*, 1998; Cebula *et al.*, 2013; Derkow *et al.*, 2011; Wuensch *et al.*, 2006). Using the transgenic mouse model Ova x Cre in which antigen presentation is restricted exclusively to hepatocytes (Sandhu *et al.*, 2011) it was demonstrated that local intrahepatic priming of adoptively transferred CD8+ T cells indeed leads to induction of immune mediated liver pathology as indicated by increased serum ALT (Fig. 9 and (Cebula *et al.*, 2013)). Since the antigen is exclusively expressed and presented by hepatocytes, a contribution of CD4+ T cells can be excluded. Thus, intrahepatic recognition of antigen leads to CD4+ independent activation and proliferation of adoptively transferred antigen specific CD8+ T cells within the liver, liver draining lymph node and spleen (Cebula *et al.*, 2013). The proliferation in lymph node and spleen is assumed to be due to the observed liver damage that enhances cross presentation of antigen by APCs within secondary lymphatic tissues. However, despite induction of intrahepatic immune responses, a reduction of antigen at the peak of liver damage was not observed (Cebula *et al.*, 2013). This might reflect an insufficient activation of antigen specific CD8+ T cells that leads to partial effector capabilities and failure against hepatic antigens as described by Derkow *et al*., 2011. The peak of ALT is assumed to reflect the highest stalling of hepatocytes. Thus, a higher immune mediated death of target cells occurs in low antigen expressing mice which might favor enhanced cross-presentation and generation of T_{eff} cells that in turn could induce antigen clearance. Clearance of low antigen started as early as day 7 upon adoptive transfer (data not shown) and was completed in all mice expressing low

amount of antigen at day 13 (Fig. 11). The kinetics of this immune response suggest a tight regulation of adoptively transferred T cells which reflects the onset of T cell responses under native conditions, taking several days to develop (Delves & Roitt, 2000a; Delves & Roitt, 2000b; Parkin & Cohen, 2001).

In summary, by transplantation of antigen specific CD8+ T cells to mice expressing antigen within hepatocytes, the present work shows the induction of CD8+ mediated immune responses within the liver. However, the outcome of intrahepatic immunity depends on the amount of antigen the T cells are exposed to. Indeed, only hepatocytes expressing low amounts of antigen can be cleared by the immune system.

It still remains to be investigated if the failure of CD8+ mediated immunity towards high antigen burden could be overcome in the presence of supporting CD4+ T_{helper} cells. Thus, on day 2 upon adoptive transfer of OT-I cells, i.e. a time point when liver pathology is induced, OT-II cells could be transferred to Ova x Cre mice expressing low or high antigen levels. This would help to investigate if the failure to clear high antigen burden is due to lack of CD4+ T cells. On the other hand, it could also be possible that the tolerogenic environment of the liver induces the development of T_{reg} which might also impair clearance of low antigen if antigen specific CD4+ T cells were present.

4.5 Antigen level dependent regulation of T cell responses

If compared to viral infection in which the concentration of peptide per cell is different, the antigen amount in the Ova x Cre model differs in the total number of antigen presenting cells. This difference depends on Tam application that leads to 50% of antigen presenting hepatocytes, while in non-treated Ova x Cre mice only 10% of hepatocytes express the antigen, due to basal activity of Cre (Fig. 4). Within the present work it could be demonstrated that the outcome of intrahepatic immunity is dependent on the number of antigen presenting hepatocytes. Importantly, while low antigen amount was cleared within 13 days, neither reduction nor clearance of high antigen was observed (Fig. 11). This failure is not due to inefficient T cell priming under high antigen conditions, since investigation of T cell proliferation on day 3 upon adoptive transfer revealed an even higher proliferative capacity of OT-I cells exposed to high antigen (Cebula *et al.*, 2013). Further the results suggest that T cell proliferation does not correlate with the outcome of intrahepatic immune responses, as a significantly less severe liver damage is observed, despite stronger T cell proliferation (Fig. 9). Rather, the results indicate a regulative role for the level of antigen itself to govern the

outcome of intrahepatic immune responses. This suggestion is evident by the observation that neither improved quality nor higher numbers of OT-I cells were able to overcome the high antigen load (Fig. 13). In both settings antigen clearance was not achieved. Although high amounts of antigen were significantly reduced (if compared to high antigen expressing mice that received 5 x 10^6 OT-I cells) the immune system could not overcome this burden. The same is true for *in vitro* pre-activated cells which did not reach a reduction of high antigen burden (Fig. 13B) and underwent deletion upon adoptive transfer (data not shown). This finding is remarkable since the pre-activated cells were considered to have gained an effector phenotype and were producing a high amount of the effector cytokines IFNy and TNFα that contribute to the eradication of intracellular antigen (Harty *et al.*, 2000; Harty & Badovinac, 2002; Wohlleber *et al.*, 2012).

The present study further shows that the course of immunity is governed by the amount of antigen presenting target cells: T cells developed an exhausted phenotype if exposed to high amount of antigen, as indicated by significantly increased expression of PD-1 and Lag-3 (Fig. 15). Indeed, the expression of PD-1 and Lag-3 was previously demonstrated to correlate with T cell exhaustion during several chronic infections (Barber *et al.*, 2006; Isogawa *et al.*, 2013; Paley *et al.*, 2012; Wherry *et al.*, 2007). Additionally, exhaustion of antigen specific T cells was reported by progressive loss of effector cytokine production and deletion of antigen specific T cells (Fuller & Zajac, 2003; Virgin *et al.*, 2009; Wherry *et al.*, 2003; Zajac *et al.*, 1998). These characteristics could also be demonstrated in this work, when antigen specific T cells were exposed to high antigen loads within the liver. In contrast, if T cells were exposed to low amounts of antigen, antigen specific T cells were maintained at the primary site of antigen exposure and secondary lymphatic tissue (see chapter: 3.3.4 Antigen specific T cells are maintained upon clearance of antigen presenting hepatocytes). Moreover, T cells exposed to low amounts of antigen showed the potential to release effector cytokines and to perform killing of antigen presenting target cells within the periphery. These results suggest the establishment of a full functional memory population when T cells are primed by low amounts of intrahepatic antigen, as indicated by expression of the memory marker CD44 (Lau *et al.*, 1994). This is in line with other reports which observe the establishment of memory cells in response to low levels of antigen, while high amounts seem to drive development of short-lived effector T cells (Badovinac *et al.*, 2004; Joshi *et al.*, 2007). Of note, SIINFEKL-tetramer specific T cells were still detected within the liver of low antigen expressing mice on day 90 upon adoptive transfer (data not shown), i.e. a time point when contraction of effector T cells has already taken place upon eradication of antigen (Oehen *et al.*, 1992; Razvi & Welsh, 1995).

Yet, although these characteristics suggest the establishment of a memory population, the exact phenotype of these cells remains to be determined. It would be interesting to investigate if these cells were of the central memory phenotype (T_{CM}) or of the effector memory phenotype (T_{EM}). If compared to T_{CM}, T_{EM} release effector cytokines such as IFNy and display only low capacity to proliferate. A deeper phenotypic characterization of the remaining cells within Ova x Cre mice upon antigen clearance might shed light into the observation of failed antigen clearance by *in vivo* generated T cell protection.

In summary, the results of the present work demonstrate that both, the course and outcome of intrahepatic immune responses depend on the number of antigen presenting hepatocytes. While high numbers seem to drive T cell exhaustion, low numbers are cleared by the immune system and allow the establishment of memory T cells.

4.6 The amount of antigen is the cause for T cell exhaustion

In this work it was demonstrated that the number of antigen presenting hepatocytes determines the outcome of intrahepatic T cell responses. The observation that the immune response is the better the lower the antigen load is supported by various reports. Indeed, both, experimental data and clinical implications have suggested an inverse correlation of the amount of antigen with the outcome of immune responses. In clinical studies on chronically HIV infected patients, successful reduction of viral load by antiviral therapy revealed improved T cell function (Day *et al.*, 2007; Streeck *et al.*, 2008). A clinical investigation on the course of HCV infection suggested a correlation of viral clearance to a low inoculum of viral particles (Thimme *et al.*, 2001). Additionally, *In vitro* cultures of antigen specific CD8+ T cells from human demonstrated a critical threshold for the antigen. While T cells exposed to low and intermediate levels of antigen showed cytolytic activity, an unresponsive state was acquired in response to high concentrations of antigen (Oved *et al.*, 2007; Wolchinsky *et al.*, 2014). Together, these data imply that it is indeed the initial amount of antigen that governs the outcome of immune responses. Moreover, an *in vitro* model for T cell priming demonstrated a mechanism in which virus specific CD8+ T cell function is influenced by the amount of antigen (Gehring *et al.*, 2007). In this system naturally HBV infected human hepatocytes were co-cultivated with HBV specific CD8+ T cells. While high amounts of HBV led to increased production of IFNy and TNFα, limited amounts of antigen stimulated T cell degranulation. These data imply different regulative mechanisms depending on the antigen amount, yet do not consider the more complex *in vivo* conditions of the unique liver milieu which might affect T cell responses. Hence, the present work demonstrates that different

amounts of antigen govern CD8+ mediated T cell responses *in vivo*. Interestingly, this phenomenon is not restricted to the liver, since studies employing a chronic LCMV infection model reported a specific impairment of T cell responses towards elevated levels of antigen (Mueller & Ahmed, 2009; Richter *et al.*, 2012). In these settings, the authors restricted antigen presentation to dendritic cells by deleting MHC class I molecules on other cell types. During the initial phase, improved T cell function was observed, as indicated by higher proliferative capacity and cytokine release. This improved function might be explained by an optimal stimulation of T cells via professional APC. However, according to the chosen settings, the antigen could not be eliminated and increased continuously. As a consequence, the efficiently induced effector T cells were overwhelmed by sustained exposure to the antigen and became severely exhausted (Mueller & Ahmed, 2009; Richter *et al.*, 2012). These results suggest that it is the amount of antigen and not the quality of T cells which drives T cell exhaustion and thus, persisting antigen. In line with these data, the present study implies a role for the antigen and not the milieu of T cell priming to be the key modulator of T cell responses.

Taken together, the regulative capacities of the antigen load towards immune responses suggests the presence of a regulative feedback loop by the immune system. In case of high antigenic amounts the immune system rather seems to become exhausted and as a consequence allows the establishment of persistent infections. This might be of importance to accept persistence of the pathogen instead of increased tissue destruction which could cause a systemic failure. Thus, the immune system tolerates the pathogen and just performs partial control to impair systemic spread of the pathogen. Such a regulative mechanism indicates the presence of CD4+ T_{reg}. However, the contribution of these cells in the regulation of intrahepatic immune responses in the current model requires further investigation.

4.7 Limitation of T cell memory and development of therapeutics

Upon efficient clearance of a pathogen by CD8+ T cells, the majority of T_{eff} undergoes apoptosis and a few antigen specific T cells remain to establish a memory population (Mueller *et al.*, 2013). Indeed, upon clearance of low antigen, the establishment of a T cell memory population was speculated. Moreover, these cells demonstrated immune protection upon peripheral challenge by an *in vivo* CTL, as demonstrated by specific killing of antigen pulsed target cells (Fig. 17). It was speculated if these T cells could also perform effector function against re-occurring antigen within the liver. Thus, T cells maintained upon clearance of low antigen were subsequently challenged with high intrahepatic antigen load

(Fig. 18). The high expression of CD44 indicates an efficient generation of memory cells (Fig. 19A). Surprisingly, the cells failed to react against the high antigen load as indicated by unaltered antigen amount (Fig. 20) and developed an exhausted phenotype. However, the T cells were not deleted as known from naïve cells exposed to high antigen amount. Rather, the maintained T cells are recruited into the liver and seem to remain there within an anergic state as they do not perform specific killing of target cells in the periphery. These data suggest that high amounts of intrahepatic antigen have not just a negative impact on naïve, but also memory cells.

It remains to be elucidated how fulminant infections, especially those with an initial high inoculum and chronic progression, e.g. hepatitis, malaria or tuberculosis, can be controlled by the immune system and may lead to cured infection. The findings of the present work reflect a specific attention for the development of therapeutic vaccines against hepatotropic pathogens. In case of HBV infection a preventive vaccine already exists, but has been shown to be ineffective in the chronic state of infection (Rehermann, 2013). Indeed, such a limitation is reflected within the present work as efficiently generated memory cells fail to overcome the high antigen load.

4.8 Immune regulation during persistent infection

T cell non-responsiveness towards antigen is characterized by either induction of anergy or exhaustion. The differentiation of these two states is not that strict, as both mechanisms allow the persistence of the antigen. However, anergy of antigen specific T cells is often associated with suboptimal priming of T cells by professional APC under homeostatic conditions to impair autoimmunity (Chung *et al.*, 2013; Wolchinsky *et al.*, 2014). In contrast, T cell exhaustion frequently takes place under pro-inflammatory conditions, when infectious pathogens are present. During chronic viral infections exhausted CD8+ T cells have been demonstrated to undergo extensive activation and cellular division due to persisting antigen (Casazza *et al.*, 2001; Shin *et al.*, 2007; Wherry *et al.*, 2004). This division is mediated in an antigen dependent manner and contrasts to the maintenance of conventional memory T cells which are generated upon acutely resolved infection. Based on both, chronically infected mice and HCV infected patients it became evident that the maintenance of exhausted T cells is controlled by the T-box transcription factor Tbet. The Tbet mediated maintenance of exhausted T cells establishes a progenitor pool of antigen specific T cells (Paley *et al.*, 2012). Although a full eradication of the virus was not achieved, the authors speculated that these cells were maintained to control viral spread by induction of steady state conditions

between virus and immune system. Such steady conditions would protect the host on the one hand from overwhelming high viral titers and on the other hand by enhanced immune destruction of the infected tissue that might end in life threatening conditions for the host. Hence, it seems as if the immune system has the decision to either resolve an infection or to become exhausted in a self-protective mechanism (Paley *et al.*, 2012). However, such a progenitor population was not observed in the present study. This might be due to the chosen conditions, since antigen specific T cells were adoptively transferred and did not derive from the endogenous precursor pool. The adoptive transfer of OT-I cells might lead to a rapid activation of all provided cells within a short while and drive their deletion.

In addition to the supposed self-regulation of effector T cells during chronic infection, one has to consider the induction of regulatory immune cells. Indeed, liver infection with HCV has been demonstrated to correlate with both, induction and proliferation of T_{reg}. Moreover, the amount of T_{reg} positively correlated to viral replication in HCV patients (Cabrera *et al.*, 2004; Manigold *et al.*, 2006; Sugimoto *et al.*, 2003). This suggests that T_{reg} play a critical role in the progression of viral hepatitis by impairment of CD8+ mediated immune responses. However, analysis of the mouse model Ova x Cre during this study did not reveal an induction of this immune suppressive cell type, as indicated by the absence of CD4+ CD25+ FoxP3+ cells within the liver of mice (data not shown). This suggests that the observed failure of antigen clearance in high antigen expressing Ova x Cre mice is mediated by the amount of antigen and not affected by regulatory immune cells. However, the results reported here require a deeper characterization of other immune regulatory mechanisms that might impair clearance of high intrahepatic antigen load.

The current work shows that not only naïve, but also antigen experienced T cells display a failure to overcome high concentrations of antigen in the liver (Fig. 13 andFig. 20). Remarkably, this failure is not restricted to the liver, but results in systemic non-responsiveness of antigen specific T cells, as indicated by *in vivo* CTL experiments (Fig. 21). Upon clearance of low antigen levels, Ova x Cre mice are supposed to establish a memory population. If compared to naïve cells, memory cells require only little amounts of antigen to become reactivated and additionally, re-activation can also take place in peripheral tissue. The lack of potent immune response when T cells re-encounter high levels of intrahepatic antigen suggests the presence of other regulative mechanisms to control CD8+ mediated immunity in the liver. Therefore, it would be important to investigate the contribution of the different cell subsets within the liver. LSEC for example, have been initially described as cells that induce an unresponsive state of CD8+ T cells (Diehl *et al.*, 2008; Limmer *et al.*, 2000). Later, LSEC were demonstrated to efficiently generate liver primed memory T cells that

display a T_{EM} phenotype, if T cell priming takes place during non-inflammatory conditions (Bottcher *et al.*, 2013). Although these findings seem to be contradictory, they suggest that LSEC display a significant potential to influence the outcome of intrahepatic immunity. Thus, they might also exert suppressive function in the current model and modulate the capacity of OT-I cells within high antigen expressing Ova x Cre mice. In this line, enhanced expression levels of *LSECtin*-mRNA, a protein which is suggested to impair intrahepatic T cell immunity (Liu *et al.*, 2013) - were significantly increased in total liver tissue of Tam treated Ova x Cre mice, as indicated by qRT-PCR (data not shown). These results suggest a contribution of LSEC to the observed failure of antigen specific CD8+ T cells to clear high antigen loads.

4.9 Conclusions

In summary, by using the transgenic mouse model Ova x Cre, the present work revealed new insights into intrahepatic mediated CD8+ T cell responses. Different amounts of antigen allow both, the activation of antigen specific T cells accompanied by the induction of tissue damage displaying a transient course. However, the severeness of tissue damage correlates inversely to the amount of presented antigen. Low numbers of antigen presenting cells can be cleared by the immune system, leading to establishment of a protective, systemic immunity. In contrast, high amounts of antigen result in activation of CD8+ T cells, but subsequently induce severe exhaustion of responding T cells. These findings might reflect the challenge of current therapy to fight pathogens that often establish a chronic state of infection and viral persistence.

5. Future perspectives and outlook

The unique feature of stochastic antigen expression in either 10% or 50% of hepatocytes illuminated a specific role for the concentration of antigen to regulate liver immunity. Potent immune responses were only established upon clearance of low antigen loads and T effector cells were exhausted when re-challenged with high antigen loads in the tolerogenic environment of the liver. It was assumed that this was a regulatory feedback loop to avoid life threatening tissue destruction. Thus, it would be interesting to investigate if the impairment of T effector cells is restricted to the liver or if it also takes place in different immune suppressive environments. Tumors are an aggressive tissue in which different danger signals, so called tumor associated antigens, are established that induce immune responses. However, tumors are known to establish a suppressive microenvironment to avoid destruction by responding T cells. Thus, upon clearance of low intrahepatic antigen, Ova x Cre mice could be challenged by transplantation of Ova expressing tumor cells to create an immune suppressive environment apart from the liver.

If compared to models that rely on infection, a significant advantage of the Ova x Cre system is that the amount of antigen is controlled and does not increase by time. Thus, CD8+ T cells are challenged by two defined conditions which might be useful to determine molecular pathways that are involved in the development of memory T cells, tolerant T cells, anergic T cells or even exhausted T cells. To investigate molecular pathways that subsequently regulate the fate of T cells upon exposure to antigen or antigen presenting cells that express low or high levels of target peptide, adoptively transferred T cells could be re-isolated from Ova x Cre mice expressing high or low antigen on different time points upon transfer. Investigation of the transcriptome from these cells might help to understand the interplay of different molecules in early, intermediate and late stages of T cells that are committed towards a specific phenotype. The results of such a transcriptome analysis might allow to find molecules that enable a precise discrimination of different T cell subsets (e.g. anergic vs. exhausted). Further, they might help to develop therapeutical approaches. One therapeutical approach for example is the administration of antibodies that block co-inhibitory ligands on target cells to restore function of exhausted T cells (Fisicaro *et al.*, 2012; Isogawa *et al.*, 2013). If transcriptome analysis would allow the determination of the exact time point when such co-inhibitory molecules are expressed, blocking of the interactions could already be performed at the onset of immune response. This might even avoid the induction of T cell exhaustion.

6. Material

6.1 Equipment

Tab. 1: List of laboratory equipment.

Name	Company
Autoclave	Belimed steam sterilizer 6-6-6 HS1 FD
Centrifuges	Heraeus Biofuge fresco
	Beckmann GS-15R
	Sigma 3K20
	Heraeus Megafuge 1.0
	Thermo Scientific Jouan CR412
	Inflexible Rotors GSA, GS3, SS34
	Swing Rotor HB4
Cell acquisition	BD™LSRII analyzer
	BD™Fortessa analyzer
	BD™ARIA cell sorter
Cell counter	Fuchs-Rosenthal
	Neubauer improved
	Guava (Millipore)
Deionized water	Millipore MilliQ
Feeding gavage	Heiland Vet GmbH
Forceps	Curved forceps, tissue forceps
Gel electrophoresis	Gibco BRL horizontal Gel
Homogenizer	MP FastPrep®-24 Lysing Matrix

LightCycler®480 for Real Time PCR	Roche
Microliter pipettes	Gilson
Microscopes	Leica Labovert FS
	Nikon TMS
	Olympus BX51TF + Color view camera Illu
Microwave oven	Whirlpool
PCR cycler	T3 Thermo cycler, Biometra
pH-meter	Beckman M340
Photometer nanodrop	Spectrophotometer, Peqlab ND-1000
Perfusion needles Vasuflo 23G	Dispomed (40023)
Pipetboy	Pipetboy IBS Integra Biosciences
Power supplies	Biotec-Fischer Phero-STAB 550
	Biorad Power PAC 200
	Gibco BRL ST 504
Precision scale	Sartorius
Scissors	Tissue scissors
Shaker	Heidolph
Steel sieves	
Sterile clean benches	BDK-SK 1500
	Sterile Guard Class II Type A/B3, Baker Company SG 400E
	Heraeus, Herasafe, Heraeus HSP 18

	Heraeus, Herasafe KS15
Table top centrifuges	Eppendorf 5417C
	Eppendorf 5436
	Heraeus Biofuge 13
Thermomixer	Heraeus Biofuge PICO 17
	Eppendorf Thermomixer compact
U.V. trans illuminator	Hanau
Vortexer	Vortex Genie 2, Scientific Industries
Water bath	GFL
Xenogen IVIS 200	Caliper Life Sciences
4°C refrigerator	Liebherr
-20°C freezer	Liebherr
-80°C freezer	Thermo Forma

6.2 Chemicals and antibiotics

Tab. 2: Overview of chemical compounds used in this work.

Compound	Company	Catalogue no.
Agarose (LE)	Biozym	840004
Ampicillin	Sigma	
BSA, molecular biology grade	NEB	B9000S
Brefeldin A	eBioscience	00-4506
Carbon tetra chloride (CCl_4)	provided by Prof. Lars Zender, HZI	
ClinOleic 20 % lipid emulsion	Baxter	2573668

Direct PCR tail lysis reagent	Peqlab	32-102-T
Easycoll, density 1,124 g/ml	Biochrom KG	L 6145
EDTA	Roth	
Glutamine	Serva	
HCl	Roth (37%)	
Heparin	Ratiopharm	3029843
HEPES	PAA	
Ionomycin	Sigma	I0634-1MG
Isofluran	Allbrecht GmbH	A29951,0025
Lipofectamine 2000	Life Technologies	11668030
Luciferin	PJK	
MEM	Gibco	61 100-087
Midori Green	Nippon Genetics	MG04
Nuclease free H_2O	Promega	
Phorbol 12-myristate 13-acetate	Sigma	P8139-1MG
Proteinase K	Qiagen	19133
SIINFEKL peptide (10 mg/ ml)	Peptide- and Chemical Synthesis Group HZI	
Streptomycin	Serva	
Sucrose	Roth	
Tamoxifen (10 mg, 30 mg)	Ratiopharm	9394.00.00 (Zul. Nr.)
TEP (10 x)	Biochrom	
Tris	Roth	
Trypanblue	Sigma	T8154-20ML93595

Viacount solution	Millipore

6.3 Kits

Tab. 3: List of complete application kits.

Name	Company	Catalogue no.
Anti-rat/mouse FoxP3 staining Kit	eBioscience	72-5775-40
BD FACS lysing solution	Becton, Dickinson	349202
Biomix	Bioline	302004
CD8a+ Tcell isolation Kit II	Miltenyi, Biotec	130-095-236
CellTrace CFSE cell proliferation Kit	Beckman Dickinson	554714
Cytofix/Cytoperm™	Beckman Dickinson	349202
LIVE/DEAD® Fixable Near IR Dead cell stain kit	Invitrogen	L10119
Nucleobond extra maxi	Macherey-Nagel	
QIAquick Gel extraction Kit	Qiagen	28706
QuantiTect SYBRGreen PCR Kit	Qiagen	204143
Ready-To-Go™ You Prime first strand beads	GE Healthcare	27-9264-01
RNase free DNaseI 79254	Qiagen	
RNeasy Mini Kit (250) 74106	Qiagen	
RNeasy Micro Kit (50) 74004	Qiagen	
TOPO TA cloning®	Invitrogen	K4500

6.4 Antibodies

Tab. 4: Overview of antibodies used. Abbreviations: FC= FlowCytometry, WB= Western blot. For FC, all antibodies were diluted in FACS-buffer.

Epitope	Conjugate	Dilution	Application	Company	Catalogue No.
CD3	FITC	300	FC	eBioscience	11-003
CD4	eFluor450	1000	FC	eBioscience	48-0041
CD8	unconjugated	100 µg i.p.	CD8 depletion	Dr. S. Weiss	HZI
CD8	APC	600	FC	eBioscience	17-0081
CD8	PE	200	FC	eBioscience	12-0081
CD8	PerCP Cy5.5	400	FC	eBioscience	45-0081
CD16/ CD32	unconjugated	500	FC/ blocking	Dr. S. Weiss	HZI
CD25	APC	500	FC	eBioscience	17-0251
CD44	APC	1000	FC	eBioscience	17-0441
CD62L	APC	4000	FC	eBioscience	17-0621
CD62L	PE-Cy7	1500	FC	eBioscience	25-0621
CD69	FITC	300	FC	eBioscience	11-0691
CD69	PE-Cy7	300	FC	eBioscience	25-0691

CD90.1 (Thy1.1)	APC	600	FC	eBioscience	17-0900
CD90.1 (Thy1.1)	PE	2000	FC	eBioscience	12-0900
CD223 (Lag-3)	PE	300	FC	eBioscience	12-2231
CD279 (PD-1)	FITC	300	FC	eBioscience	11-9985
CD279 (PD-1)	PE	300	FC	eBioscience	12-9985
Ova MHC class I H2Kb	APC	500	FC	eBioscience	17-5743
Chicken egg Ovalbumin SIINFEKL	unconjugated	-	WB	Sigma Aldrich	A6075
SIINFEKL tetramer	PE	200	FC	Beckmann/ Coulter	T03000

6.5 Buffers media and solutions

Tab. 5: Overview of buffers media and chemical solutions used in the present work.

Substance	Ingredients or company no.
ß-Mercaptoethanol (1000x)	100 mM ß-ME (v/v) in ddH_2O
Erythrocyte lysis buffer	150 mM NH_4Cl
	10 mM $KHCO_3$
	0,1 mM EDTA
	pH 7,2-7,4
FACS buffer	PBS
	2% (v/v) FCS
FCS (fetal calf serum)	JRH Bioscience, Biowest
Glutamine (100x)	29,23 mg/ml Glutamine
	sterile filtered
HEPES (1 M)	283,3 g
	pH 7,2
	ad H_2O 1 l
Liver digest medium	Gibco Nr. 17701-034
Liver perfusion medium	Gibco Nr. 17703-038

MACS buffer	PBS
	1% (v/v) FCS
MEM	Minimum essential medium
	100 ml HEPES (1 M)
	8,5 g $NaHCO_3$
	pH 7-7,4
	ad H_2O 10 l
Penicillin/Streptomycin (100x)	6,06 mg/ml ampicillin (10000 U/ml)
	10 mg/ml streptomycin
	sterile filtered
	pH 7,4
	ad H_2O 1 l
PBS	140 mM NaCl
	27 mM KCl
	7,2 mM Na_2HPO_4
	pH 6,8- 7
	ad H_2O 10 l

RPMI	Roswell Park Memorial Institute medium; RPMI powder
	100 ml HEPES (1 M)
	1,68 g/l $NaHCO_3$
	pH 7- 7,4
	ad H_2O 10 l
Trypsin-EDTA	100 ml TEP-PBS (10 x)
	ad H_2O 1 l

6.6 Enzymes

6.6.1 Restriction enzymes

Tab. 6: List of endonucleases that were used within the present work.

Name	Stock concentration	Company	Catalogue no.
ApaLI	10000 U/ml	NEB	R0507
BamHI (high fidelity)	20000 U/ml	NEB	R3136
BglII	10000 U/ml	NEB	R0144
BssHII	4000 U/ml	NEB	R0199
ClaI	5000 U/ml	NEB	R0197
EcoRI (high fidelity)	20000 U/ml	NEB	R3101
MfeI	10000 U/ml	NEB	R0589
NaeI	10000 U/ml	NEB	R0190
NcoI (high fidelity)	20000 U/ml	NEB	R3193
NruI	10000 U/ml	NEB	R0192
PstI	20000 U/ml	NEB	R0140
PvuII	10000 U/ml	NEB	R0151
SacII	20000 U/ml	NEB	R0157
StuI	10000 U/ml	NEB	R0187

6.6.2 Enzyme buffers

Tab. 7: List of buffers used for endonucleases as indicated in Tab. 6.

Name	Concentration	Company	Catalogue no.
NEBuffer 1	10 X	NEB	B7001S
NEBuffer 2	10 X	NEB	B7002S
NEBuffer 3	10 X	NEB	B7003S
NEBuffer 4	10 X	NEB	B7004S

6.6.3 DNA molecular size markers

Tab. 8: Tabular overview of molecular DNA markers used within the present study.

Name	Size	Company	Catalogue no.
Hyper ladder I	200-10000 bp	Bioline	Bio-33053
Hyper ladder V	25-500 bp	Bioline	Bio-33057

6.7 Consumables

Tab. 9: Overview of expendable materials used within the present work.

Consumable	Company
Bacterial petri dish	TPP
Cannulas (26 gauge)	Braun
Cell culture flasks (25 cm^2, 175 cm^2, 300 cm^2)	Nunc, TPP
Cell culture plates (6 well, 24 well, 96 well)	Nunc, Corning
Cell strainer 100 µm Nylon	BD Falcon
Combi Tips (0,5 ml, 1,25 ml, 2 ml, 5 ml)	Eppendorf

Cryogenic vials	CytoOne
Falcon Tubes (15 ml, 50 ml)	Greiner BioOne
Gloves (nitrile, latex)	Microflex
Haematokrit capillaries (with heparin)	Schmidt
LS columns	Miltenyi Biotec
Lysing matrix D	MP Biomedicals
PCR tubes (0,2 ml)	Biozym
Perfusion needles (23G) Ecoflo	Dispomed
Pipette tips (10 µl, 20 µl, 200 µl, 1000 µl)	StarLab
Reaction Tubes Safe lock (1,5 ml, 2 ml)	Sarstedt
Syringes (1 ml, 2 ml, 5 ml, 10 ml, 20 ml, 50 ml)	Omnifix
Syringe filters (0,2 µm, 0,45 µm)	Sartorius
Tissue culture dish	Corning, Greiner
Tissue culture flask (25 cm^2, 75 cm^2, 175 cm^2, 300 cm^2)	Nunc,TPP
V-shape micro plate (96 well)	Nunc

6.8 Software

Tab. 10: Tabular overview of computer based analysis software.

Name	Company
Adobe Photoshop (CS5)	Adobe Systems
FACS Diva (v 5.0.2)	BD
FlowJo (v 10)	TreeStar Inc.
GeneSpring	Agiland

GraphPad Prism (v 5)	Statcon
LightCycler® 480 Software release 1.5.0	Roche
Living Image (v3)	Caliper life sciences
MS office (2010)	Microsoft

6.9 Primer

Tab. 11: Tabular overview of oligomer sequences as used during the study. Abbreviations: gt: genotyping; RT: realtime PCR.

Name	Sequence (5´-3´)	Application	Designed by
AgFusion1a	CAGGCACTCCTTTCAAGACC	RT-PCR	Dr. U. Hillebrand
AgFusion2a	CCAAAAGACGGCAATATGGT	RT-PCR	Dr. U. Hillebrand
AgFusion3a	GGAAAACTCGACGCAAGAAA	RT-PCR	Dr. U. Hillebrand
AgFusion4a	GCGGTTGAGGACAAACTCTT	RT-PCR	Dr. U. Hillebrand
Cre1	GCCTGCATTACCGGTCGATGCAACGA	gt	Dr. U. Hillebrand
Cre2	GTGGCAGATGGCGCGGCAACACCATT	gt	Dr. U. Hillebrand
EMCVneo1	AAGAGTCAAATGGCTCTCCTCAAGCGTATT	gt	Dr. U. Hillebrand
EMCVneo2	GTCTGTTGTGCCCAGTCATAGCCGAATAG	gt	Dr. U. Hillebrand
mAlbclifwd	GACAAGGAAAGCTGCCTGAC	RT-PCR	C. Lipps
mAlbclirev	TTCTGCAAAGTCAGCATTGG	RT-PCR	C.Lipps

Ovafwd1	GCGCGCGTCGACACCATGGAATTTTGTTTTGATGT	cloning	A. Ochel
Ovarev1	GGATCCTTAAGGGGAAACACATCTGC	cloning	A. Ochel
Ovafl_RT_fwd 2	GGCATCAATGGCTTCTGA	RT-PCR	A. Ochel
Ovafl_RT_rev2	TCTCAAGCTGCTCAAGGC	RT-PCR	A. Ochel
Ova sense	GTTTGAAAGTTGATAGGTC	cloning	A. Ochel
Ova as	TGAGAGTATAATCAACTTTG	cloning	A. Ochel

6.10 Plasmids

Tab. 12: Tabular overview of plasmids used within this work.

Name	Size	Source	
pEMTARantiOvaAg	4465 bp	Dr. U. Hillebrand	(Sandhu *et al.*, 2011)
pCRTOPO2.1	3900 bp	Invitrogen	

6.11 Cell lines

Tab. 13: Overview of cell lines used within the present study.

Name	Origin	Source
HEK 293 T	Large T antigen immortalized cell line from human embryonic kidney	(Graham *et al.*, 1977)

6.12 Bacteria

Tab. 14: List of bacterial strains used during the work for molecular applications. Competent bacterial strains were generated in house.

Name	
Top 10	chemically competent *Escherichia coli*
XL-1 blue	electro competent *E. coli*

6.13 Viruses

Tab. 15: Overview of viral strains used during the present work.

Name	Strain	Source
Adeno-Luc-GFP	Type AdV5 (Δ*E1*)	kindly provided by Dr. Werner Lindenmaier, HZI
MCMV Luc		kindly provided by Prof. Luca Cicin-Sain, HZI

6.14 Mice

All mice used were bred and maintained in the in house animal facilities of the Helmholtz Center for Infection Research. Mice were of the Black 6 background from Jackson laboratory, herein after referred to as BL/6, if wildtype mice are mentioned. Animals were bred and maintained under specific pathogen free (SPF) conditions in single ventilated cages. Further the mice received all nutrients *ad libitum* and had a controlled circadian rhythm of 12 hours. Animal experiments were performed in accordance to the german animal welfare law. The institutional guidelines have been approved by the local government of lower saxoney (permission: 33.14-42502-04-10/0199).

Tab. 16: Overview of mouse strains used within this study. tg: transgenic

Description	Phenotype and source
Ova	*ova* is integrated in antisense orientation into the *Rosa26* locus flanked by loxP sites (Sandhu *et al.*, 2011)
$CreER^{T2}$	Cre recombinase fused to the tamoxifen dependent estrogen receptor is expressed under control of the serum albumin locus (Schuler *et al.*, 2004)
Ova x Cre	Double transgenic animals for Ova and CreERT2, allowing tamoxifen dependent induction of the ova sense orientation exclusively in hepatocytes (Cebula *et al.*, 2013; Sandhu *et al.*, 2011). The model is based on heterozygous Ova-mice that carry the antisense orientated SWINFEKL encoding sequence of *ovalbumin* under control of the ubiquitously active *rosa26* promoter. The transgene differs in one amino acid to the immunogenic wildtype protein of $Ova_{SIINFEKL}$.

OT-I (Thy1.1)	These mice are transgenic (tg) for CD8+ T cells which express the Vß5.1 chain for the SIINFEKL specific T cell receptor (TCR). According to thymic development the SIINFEKL specific α chain of the TCR develops leading to a full functional TCR that recognizes the Ova epitope SIINFEKL in context of MHC class I molecules. In addition the cells expressed the congenic marker Thy1.1 (CD90.1) which allows backtracking upon adoptive transfers as Bl6/J express Thy1.2.
OT-II (Thy1.1)	These mice are transgenic for CD4+ T cells which express the Vß5.1chain for the ISQAVHAAHAEINEAGR specific TCR. OT-II mice were originally described in (Barnden *et al.*, 1998). OT-II mice used within this study were kindly provided by Prof. Carlos A. Guzman.
OT-II x Ova x Cre	These triple transgenic animals were generated by mating OT-II tg mice to Ova x Cre double tg mice and allow the investigation of CD4+ T cell responses towards the auto antigen Ova.
Bl6/J	Black 6 inbred wildtype inbred strain. These mice were used for in vivo CTL experiments as donors to isolate antigen presenting cells from the spleen.

7. Methods

7.1 Sterilization of equipment, media and solutions

Buffers for gel electrophoresis and media were produced using autoclaved deionized water exclusively. All solutions, plastic consumables and surgical instruments were sterilized by autoclaving for 25 min. at 121°C. In case of heat sensitive components, solutions were sterilized by filtration using a filter with 0,2 µm pore size. Glassware was sterilized for 4 h at 180°C in a cabinet drier.

7.2 Molecular biological methods

7.2.1 Work with nucleic acids

Nucleic acids were stored at -20°C in TE-buffer or deionized H_2O. RNA was stored in RNase free H_2O (Qiagen) at -20°C.

TE-buffer:	10 mM	Tris/ HCl (pH 7,6)
	1 mM	EDTA

7.2.1.1 Nucleic acid analysis

Determination of DNA and RNA concentration

For quantification of nucleic acids within an aqueous solution the extinction of such a solution was measured at a spectral photometer at a wavelength of 260 nm. The extinction value of an optical density (OD) of 1 is equivalent to a concentration of 50 µg dsDNA/ ml solution and 40 µg ssRNA/ ml solution (Green, 2012). The purity of the solution was calculated by the ratios of extinction values against other wave lengths. The ratio of OD_{260}/OD_{280} is an indicator for potential contaminations with proteins. The differences to the optimal value depicted in the literature display the grade of contamination.

Literature value: $OD_{260}/OD_{280}=1,8$

To determine the concentration of DNA/RNA, 1 µl solution was directly applied to a Nanodrop (Peqlab) and its extinction was measured. As nucleic acids were either dissolved in H_2O or TE-buffer, the respective solvent was used as a blank control.

7.2.1.2 DNA preparation

Plasmids are extra chromosomal DNA elements with prokaryotic origin which are localized in the nucleoid in a supercoiled form. In addition they have specific sites for restriction enzymes and therefore allow genetic manipulation such as cloning of genes. Upon cloning of such constructs competent bacteria may be transformed with those which allow amplification of the gene of interest. By various DNA preparations the plasmids can be re-isolated from the bacterial cells. Isolation of plasmid DNA from liquid bacterial overnight (o.n.) cultures was dependent on the amount of needed DNA.

7.2.1.3 Enzymatic modifications of DNA

Digestion of DNA elements was performed using enzymatic kits (Tab. 6 and Tab. 7), according to manufacturer´s protocol. Those modifications allow the integration of PCR products into bacterial plasmids or the addition of reporter genes upstream or downstream of the gene of interest to detect the subcellular localization of a desired protein.

Restriction of DNA fragments is based on the ability of restriction endonucleases to cut at specific target sequences within the molecule of interest. The treatment with such enzymes leads, depending on the enzyme used, to the generation of blunt or sticky ends within the molecule.

The appropriate buffer, incubation time and temperature may vary and depend, on the one hand, on the amount of sequences accessible for the enzyme and on the other hand on the manufacturer´s guidelines. Generally, if not indicated otherwise, 2-3 units enzyme were used per µg DNA and incubated at 37°C for 3 h.

In this study DNA restriction was used to create vectors for *in vitro* transcription of RNA encoding the antisense orientation of *ova*. In addition, restriction enzymes were used to verify the accuracy of DNA preparation from bacteria (test digestion).

7.2.1.4 Gel electrophoresis

Gel electrophoresis is a biochemical assay which separates DNA fragments of different sizes within an electric field. Based on the negative charge of DNA, these fragments migrate to the electrode of opposed polarity. This allows the separation of DNA fragments according to their size only. This effect is due to the migration potential of molecules with different sizes as small molecules have a higher migration capacity than large molecules.

This method of separation was used for analytical and preparative purposes. Analytical gels were performed to analyze PCR products and DNA fragments following restriction digests. Furthermore, they were done to proof the accuracy of *in vitro* transcription products. Preparative gels were performed to clone *ova* into the backbone the vector plasmid pCRTOPO2.1. In both cases agarose was used as gel matrix.

Agarose gels

Analytical agarose gels

Agarose gels can be used as matrices to separate DNA molecules with a size of >100 bp (Southern, 1979). The accuracy of separation within those gels can be adjusted by varying the concentration of agarose (0.8 -2 % w/v). Agarose gels were produced by resuspension of the required amount of agarose in 1 x TAE buffer and subsequent heating of the solution. To visualize the DNA, Midori Green was added directly to the agarose solution. Upon polymerization of the gel matrix, 1 x TAE buffer was added to the chamber, the samples were diluted 1:5 with 6 x loading buffer and loaded onto the gel. For the analysis of PCR products amplified by use of Biomix (see chapter: 7.2.3.1 cloning and genotyping) the reaction was loaded onto the gel without prior application of loading buffer. To have a comparison of fragment size, 2,5 µl of a DNA molecular size standard were used and separated in a discrete lane. The standard used was dependent on the expected size of DNA fragments. For large fragments DNA Hyperladder I was used, for small molecules DNA Hyperladder V (see Tab. 8).The amperage provided was dependent on the concentration of the gel matrix. For gels with a concentration of ≥0,8% (w/v) 100-120 V were provided. The documentation and visualization of DNA fragments were performed using a UV-trans illuminator equipped with a camera system.

Preparative agarose gels

Preparative gels were performed to isolate specific fragments from a mixture of fragments, e.g. if specific fragments of DNA were required after restriction digestions. Following separation of DNA fragments within the agarose matrix, the fragment of interest was detected using a trans illuminator and cut out of the agarose gel using a sterile scalpel. The extraction of the DNA within the desired band was conducted using a complete kit (QIAquick Gel extraction Kit, Qiagen) according to manufacturer´s protocol. The extracted band was then eluted in 20 µl ddH_2O instead of TE-buffer. In contrast to analytical gels, in which only small amounts of DNA are separated, in preparative gels the total volume of the desired reaction was used. This ensures a maximum yield of DNA for further steps.

50 x TAE buffer	2 M Tris/ HCl (pH 8.0)
	50 mM EDTA
	adjust pH 8,0 using glacial acetic acid
6 x Gel loading buffer	40% (w/v) Ficoll
	0,05% (w/v) Brominephenolblue
	0,05% (w/v) Xylencyanol

7.2.1.5 Ligation

Ligations are reactions in which 5´phosphate groups and 3´hydroxygroups of dsDNA molecules are covalently linked generating continuous DNA strands. This reaction requires ATP to catalyze these phospho diester linkages. Ligations were performed in order to clone restriction fragments into plasmid backbones.

Assay	Vector backbone	1 µl
	Insert	7 µl
	10 X Ligase buffer	2 µl
	T4 DNA ligase	1 µl
	ad H_2O	20 µl

The ligation mixture was incubated o.n. at 16°C and subsequently transformed into electro competent bacteria (XL-1 blue) as described in chapter: 7.3.3 Transformation of competent bacteria. The DNA was isolated the next day using a mini preparation (see chapter: 7.2.1.6 DNA purification).

7.2.1.6 DNA purification

In order to obtain plasmid DNA for molecular biological techniques as well as the desired DNA construct after cloning, DNA was purified from bacteria according to the protocol described below. Further, chromosomal DNA was isolated from eukaryotic tissue for genotyping of transgenic mice.

DNA small scale isolation (mini preparation)

To extract plasmid DNA from bacteria, 2 ml LB medium supplemented with 50 µg/ml of the required antibiotic were inoculated with the respective *E. coli* clone and cultured o.n at 37°C while shaking at <600 rpm. The next day these o.n. cultures were transferred to a centrifugation tube and centrifuged for 1 min. at 13000 rpm. The supernatant was discarded and the pellet resuspended in 500 µl STET-buffer by thorough vortexing. 50 µl lysozyme/TE (10 mg/ml) were added to lyse the bacterial cell wall and the reaction mixture was incubated at room temperature (RT) for 2 to 3 min. To inactivate the lysozyme, the assay was heated for 2 min at 95°C and centrifuged for 5 min. at 13000 rpm. The resulting pellet was removed, using an autoclaved tooth picker, 50 µl 8 M NH_4Ac as well as 500 µl isopropanol were added to the supernatant containing the DNA. The suspension was centrifuged at 13000 rpm for 5 min. to precipitate the DNA. The supernatant was discarded and the DNA pellet air dried at RT. Afterwards, the DNA pellet was resuspended in 50 to 100 µl TE-buffer, supplemented with RNase and incubated at 37°C until the DNA was completely dissolved. 5 µl of purified DNA were used for a test restriction to verify the correct plasmid DNA (see chapter: 7.2.1.3 Enzymatic modifications of DNA).

STET-buffer	Sucrose	80 g/l
	Triton X 100	0,5%
	EDTA	50 mM
	Tris/HCl	10 mM
	Adjust pH 8,0	
Lysozyme	Lysozyme in TE buffer	10 mg/ml
TE RNase	RNase A in TE buffer	10 µg/ml

7.2.2 Work with RNA

The expression of a gene is mediated by binding of the RNA polymerase II (RNA Pol II) to the promoter region of a given gene. The RNA Pol II drives along the ORF of this gene, copies its nucleotide sequence and thereby processes a transcript called, messenger RNA (mRNA) until it reaches a stop codon which leads to termination. The mRNA is further processed and then released into the cytoplasm where its sequence can be translated into a polypeptide. The presence and abundance of mRNA´s within a cell can give knowledge about the expression status and quantity of a specific gene and thus, it is an indirect proof, if a gene is expressed under specific conditions on a cellular level. The presence of transcripts from a specific gene can be shown by marking them specifically. Further, the amount of RNA molecules from a specific gene allows quantifying the strength of gene expression. This can be done by qRT-PCR as well as whole mount *in situ* hybridization (WISH).

7.2.2.1 RNA purification and storage

Within this study, the purification of RNA was performed using the RNeasy kit from Qiagen according to the manufacturer´s instructions. The purified RNA was eluted in 30–50 µl RNase-free H_2O. To avoid contamination with DNA, an on-column DNaseI I digestion was performed during the purification. In case very low amounts of cells were used, the RNA was isolated by use of the RNAmicro kit from Qiagen, in this case the RNA was eluted in 14 µl RNase-free H_2O. All RNA samples were stored at -20°C for short-term use. For long-term storage the samples were kept at -70°C.

RNA isolation upon *in vitro* transcription

The purification of *in vitro* transcribed RNA was performed using the RNeasy kit from Qiagen. Upon *in vitro* transcription RNA was purified and subsequently used for whole mount RNA *in situ* hybridization (WISH). The *in vitro* transcription mix was filled with RNase-free H_2O to a total volume of 100 µl. Then, 350 µl RLT and 250 µl EtOH (100%) were added to the mixture and the whole suspension was transferred to an RNeasy Spin column. Following centrifugation at 13000 rpm for 1 min., the flow through was discarded. The column was washed twice with 500 µl RPE buffer and centrifuged at 13000 rpm for 1 min. for each step. To dry the column, it was centrifuged for 5 min. at 13000 rpm and then transferred to a new, RNase-free centrifugation tube. 50 µl RNase-free H_2O were directly added to the center of the column and incubated for 2 min. followed by elution of the bound DNA by centrifugation at 13000 rpm for 1 min. The purified RNA was stored at -20°C.

RNA isolation from liver tissue

RNA was isolated from murine liver tissue to subsequently generate complementary DNA (cDNA). This cDNA was used in qRT-PCR to determine the amount of antigen expression within the liver of Ova x Cre mice.

Liver tissue was stored at -70°C and thawn on ice directly before RNA isolation. The sample was transferred to a FastPrep tube (Lysing matrix D, MP Biomedicals) containing 600 µl RLT buffer supplemented with 1/10 ß-ME. The whole tissue was homogenized 3 times for 20 sec. in a tissue homogenizer. Afterwards the RNA was purified using the RNeasy kit (Qiagen): The disrupted tissue was centrifuged at 13000 rpm for 3 min. and the supernatant transferred to another tube containing 1 volume 50% ethanol. The sample was mixed and then, a maximum volume of 700 µl was loaded to an RNeasy column (Qiagen). The column was centrifuged for 15 sec. at 13000 rpm and the flow through was discarded. The remaining cell extract/ethanol was loaded onto the column and centrifuged as described above. The column was washed once using 350 µl RW1 buffer by centrifugation at 13000 rpm for 15 sec. To remove remaining DNA, 10 µl DNaseI I (Qiagen) were diluted in 70 µl RDD buffer and added directly to the center of the column. The column was incubated for 15 min. at RT and washed again with 350 µl RW1 buffer (13000 rpm, 15 sec.). Afterwards the column was washed twice with 500 µl RPE buffer and centrifuged at 13000 rpm for 15 sec. or 2 min. in the last washing step. Afterwards the column was placed to a new centrifugation tube and centrifuged for 1 additional minute to remove remaining RPE buffer. Upon this centrifugation the RNA eluted in 30 µl RNase-free H_2O by spinning the tube for 1 min. at 13000 rpm. To increase the RNA yield, the eluate was loaded onto the column again and centrifuged at

13000 rpm for 1 min. 1 µl of the eluate was used to determine the concentration using the Nanodrop (see chapter: 7.2.1.1 Nucleic acid analysis). The residual sample was stored at -20°C until it was further processed.

7.2.2.2 RNA *in vitro* transcription and *in situ* hybridization

RNA *in situ* hybridization is a method by which an antisense probe is introduced into previously fixed cells. As the probe against an mRNA molecule is in antisense orientation, it binds to the specific mRNA molecule, creating a dsRNA hybrid. This dsRNA hybrid in turn can be visualized as the probe is labeled either with an antigen, recognized by an antibody, or radioactively (Almeida Engler *et al.*, 1994).

RNA *in situ* hybridization was used to analyze the percentage of *ova* expressing hepatocytes in the Ova x Cre mouse model. The used RNA probes were labeled with Digoxigenin (DIG) during *in vitro* transcription using a DIG-labeling kit, kindly provided by Prof. Reinhard Köster (Technical University Braunschweig).

Assay

Linearized template DNA (PCR Topo 2.1_Ova cut with BamHI)	10 µg
10 x Transcription buffer	3 µl
10 x DIG buffer	3 µl
T 7 RNA polymerase	1 µl
RNasin	1 µl
H_2O	*ad* 29 µl

The assay was incubated at 37°C for 1 h. Afterwards 1 µl of T-7 polymerase was added to the mixture once more and incubated for an-other hour. Upon incubation, 1,5 µl DNaseI I were added to the reaction and incubated for 20 min. at 37°C to degrade the remaining DNA template. Upon the DNaseI I digestion, the RNA was purified as described below (see chapter: 7.2.2.1 RNA purification and storage).

7.2.2.3 cDNA synthesis

The discovery of the viral enzyme reverse transcriptase (RT) (Baltimore, 1970) was initially contradictory to the central dogma of molecular biology, which so far assumed that the transfer of gene information from DNA via mRNA to a polypeptide is unidirectional (Orphanides & Reinberg, 2002). Yet, nowadays reverse transcription provides various analytical possibilities within molecular biology.

RT is an RNA dependent DNA polymerase that synthesizes a complementary DNA molecule (cDNA) based on an RNA template. The reaction is mediated by initial binding of a primer to the 3´-OH group of an RNA molecule which is then elongated by the RT, creating a dsRNA/DNA hybrid. After degradation of the RNA template strand, a dsDNA molecule is synthesized according to the sequence of the cDNA (Keller & Crouch, 1972).

Using this method, the abundance and frequency of an mRNA can be investigated by qRT-PCR (see chapter: 7.2.3.3 Quantitative Realtime PCR (qRT-PCR)). The cDNA synthesis can be performed in two ways. Either by using a specific primer, which only produces cDNA from a specific mRNA molecule, or by use of an oligo-dT primer that unspecifically binds to the poly A tail of mRNA. Thus, a cDNA is synthesized from every mRNA template that is present. Upon the RT reaction cDNA can be used directly for PCR without further purification or modification.

For quantitative RT-PCR, isolated RNA was adjusted to a concentration of 100 ng/µl and dissolved into 30 µl RNase-free H_2O. The cDNA synthesis was performed using the Ready to go you prime 1^{st} kit (GE Healthcare) according to manufacturer´s protocol. cDNA was stored at -20°C.

7.2.3 Polymerase chain reaction (PCR)

The PCR was developed in 1987 by Kary B. Mullis and is a method which allows to specifically amplify a DNA fragment of interest *in vitro* (Mullis & Faloona, 1987). The yield will give a high amount of amplicons, as the reaction leads to exponentially reactions of polymerization. The PCR is based on three general components: 1. a double stranded DNA dependent DNA polymerase, which catalyzes the amplification of DNA. As the fragment of interest that has to be amplified is ds, the DNA has to be denatured at high temperature prior to amplification, and therefore, a heat-stable DNA polymerase (*Taq* from *Thermus aquaticus*) was used. 2. a DNA fragment which is used as a template for amplification. 3. a primer pair flanking the region of interest. The primers are complementary to the sequence that has to

be amplified. Upon hybridizing to the template, primers are recognized by the polymerase. To mediate the amplification the primer has to have a free hydroxygroup at the 3´end. The polymerase binds to this hydroxygroup and starts to catalyze amplification of DNA in direction of the 3´end, a process called elongation.

The PCR´s are cyclic reactions. Each cycle is temperature dependent and divided into three steps, called denaturation, annealing and elongation. Denaturation of the template, in which the hydrogen bonds between the DNA strands are cleaved, is performed at 94°C. The temperature of this reaction depends on the GC-content of the template as these base pairs are bridged to each other by three hydrogen bonds. Annealing is the hybridization of the complementary primer to the single DNA strand and is performed at temperatures between 45°C to 65°C. The last reaction is the elongation in which the primer is extended and the new dsDNA synthesized. This step is done at 72°C which is the optimal temperature of the *Taq* DNA polymerase.

All primers used were designed using the online tool PRIMER3. The design was performed with respect to an optimal length of 17-18 nucleotides in the area of hybridization and a GC content between 50 to 60%. The required melting temperature of primers was between 55°C to 60°C. The forward and reverse primer should not share any complementary region to each other to avoid primer hybridization.

The amount of cycles varied depending on the experiment performed. For cloning and genotyping, 31 cycles were done, for realtime PCR 45 cycles were performed.

In this work PCR was used to generate PCR products which were cloned into targeting vectors or to verify the genotype of the used transgenic mice. Realtime PCR was performed to detect the amount of *ovalbumin* expressed in murine hepatocytes and to confirm antigen clearance.

7.2.3.1 Cloning and genotyping

Genotyping assay

Biomix	15 µl
fwd primer	2,5 µl
rev primer	2,5 µl
mouse tail DNA	2 µl
ad H_2O	30 µl

Amplification reactions for cloning

Template (10 ng/ µl)	
10 x reaction buffer	5 µl
DMSO	1 µl
fwd primer (10 pmol)	5 µl
rev primer (10 pmol)	5 µl
dNTP mix (25 mM)	8 µl
Polymerase	0,75 µl
ad H_2O	50 µl

The PCR cycles in genotyping and cloning reactions were similar and were performed as described as follows.

1.	95°C	5 min.	initial denaturation
2.	72°C	10 min.	
3.	94°C	30 sec.	denaturation
4.	58°C	45 sec.	annealing
5.	72°C	90 sec.	elongation go to step 2, 29 cycles
6.	72°C	10 min.	final elongation
7.	16°C	hold	

7.2.3.3 Quantitative Realtime PCR (qRT-PCR)

qRT-PCR allows the exact quantification of target nucleic acids. This is achieved by the addition of fluorescent dyes that bind into the minor groove of dsDNA molecules. As the fluorescent intensity increases proportional to the amount of generated PCR product within each cycle, it enables to conclude the amount of initial target RNA. In contrast to RNA *in situ* hybridization, which allows the analysis of gene expression pattern on a cellular level, qRT-PCR specifically detects the total amount of target sequence. Within qRT-PCR one discriminates between absolute and relative quantification. The absolute quantification determines the actual amount of a target sequence. This amount can be depicted as copy number or concentration. In contrast, the relative quantification enables a numerical estimation of a target molecule, compared to a reference gene. A so called housekeeping gene served as reference gene as its expression is not changed, despite different experimental conditions. The calculated ratio of target molecule vs. housekeeping gene can be compared to the ratio of other samples which allows the analysis of changes in the gene expression pattern under different experimental approaches.

In this work relative qRT-PCR was used to determine the expression pattern of *ova* within the hepatocytes of Ova x Cre mice upon Tamoxifen treatment, compared to non-treated Ova x Cre mice. The expression of *ova* was always compared to the hepatocyte specific gene *albumin*. Furthermore, qRT-PCR was performed to detect the clearance of Ova presenting hepatocytes.

cDNA generated during cDNA synthesis (see chapter7.2.2.3 cDNA synthesis) was diluted in nuclease free H_2O in a 1:10 ratio. The reaction was performed in the LightCyler® 480 (Roche) using a special 96 well plate (Roche). The qRT PCR for each sample was performed in triplicates.

Assay	
cDNA (1:10)	5 µl
fwd primer	2,5 µl
rev primer	2,5 µl
SybrGreen	10 µl

A mastermix consisting of SybrGreen, fwd and rev primer was prepared and given into a 96-well. To relate the gene expression of each target gene, each sample was investigated separately, performing a qRT-PCR against a housekeeping gene. The plate was centrifuged for 1 min. at 1000 rpm to allow the mastermix to settle at the bottom of the well and 5 µl of the previously diluted cDNA were added to the respective well.

The reaction was performed according to the following conditions in the light cycler

1.	Pre incubation	95°C	15 min.
2.	Amplification		
	a. Denaturation	95°C	15 sec.
	b. Annealing	58°C	20 sec.
	c. Elongation	72°C	30 sec.
3.	Melting curve		
	a.	95°C	5 sec.
	b.	70°C	1 min.
	c.	95°C	continuously
4.	Cooling	40°C	30 sec.

7.3 Work with bacteria

7.3.1 Cultivation and storage

The bacteria listed in Tab. 14 were cultivated either in LB medium or on LB agar plates at 37°C in the presence of antibiotics. In general bacteria can be stored for up to 8 weeks on agar plates at 4°C. For long-term storage a bacterial liquid culture was added to the appropriate amount of sterile 50 % glycerol and the bacterial stocks were stored at -70°C.

LB- medium:	10 g/l	Bacto Trypton
	5 g/l	Yeast extract
	10 g/l	NaCl
	Autoclaved	
Agar plates:	10 g/l	Bacto Trypton
	5 g/l	Yeast extract
	10 g/l	NaCl
	15 g/l	Agar
	autoclaved	

Autoclaved, cooled down to 50°C, supplemented with antibiotics and added to petri dishes. Stored in the dark at 4°C.

7.3.2 Generation of competent bacteria

The competence of bacterial cells is achieved by previous treatment of those with calcium or other bipolar ions. These molecules permeabilize the cellular membrane an thereby allow an incorporation of exogenous substances (Mandel & Higa, 1970). Competent bacteria were shock frozen in liquid nitrogen and stored at -80°C until they were used for transformation experiments.

7.3.3 Transformation of competent bacteria

Transformation is the transfer of plasmid DNA into competent bacteria to allow the amplification of vectors (plasmid DNA).

For plasmid amplification, 1 µl of the desired vector DNA were added to 50 µl competent bacteria (Tab. 14) and incubated on ice for 5 min. Afterwards, the cells were incubated at 37°C for 10 min. and then chilled on ice for 90 sec. Following heat shock, 1 ml LB media was added to the bacterial cells and the suspension was incubated for 30 min. at 37°C. Following this incubation period, 10 µl and 1 ml of the bacterial suspension were platted on a selection agar plate, respectively and incubated over night at 37°C. On the following day colonies were picked to inoculate liquid cultures for DNA purification (see chapter: 7.2.1.6 DNA purification DNA small scale isolation (mini preparation)) and restriction analysis to verify the accuracy of the DNA.

Following ligations, the transformation of competent bacteria was done as described above. In contrast, instead of 1 µl of plasmid DNA, the whole ligation volume (20 µl) was added to the competent cells.

Electro competent bacteria (XL-1 blue) were thawn on ice in a 1.5 ml reaction tube. 1 µl vector that had to be amplified, or complete ligation reactions were given to the cells and stored on ice. Liquid droplets were removed from the outer side of the cuvette and the bacteria with vector were transferred into it. The cuvette was placed into the chamber and pulsed with 25 µF capacity/200 ohm. The bacteria were electroporated for 4,4 s at 2,5 kV. Subsequently they were transferred into a reaction tube supplied with 1 ml LB medium and kept for 30 min. at 37°C. Afterwards the cells were plated on selection agar as described above.

7.4 Work with viruses

For the work with viruses only sterile plastic pipettes and filter tips were used. Work was performed under sterile S2 conditions.

7.4.1 Cultivation of low passage HEK 293T cells

HEK 293T cells were cultivated in cell culture flasks in MEM/5% FCS at 37°C in the presence of 95% humidity, 5% CO_2. Initially, cells were cultured in 25 cm^2 flasks. When the cells

reached a confluence of 80-90%, cells were expanded to larger flasks (175 cm^2 and finally 300 cm^2). For expansion, the medium was removed and cells were gently washed in PBS. Upon removal of PBS, cells were detached by adding the appropriate volume of trypsin to the cells. Cells were incubated in presence of trypsin at RT until detaching was visible under the microscope. Trypsin was stopped by adding MEM/5% FCS to the suspension. The suspension was gently resuspended and cells were transferred to fresh medium in a ratio of 1:10/flask.

7.4.2 Adenoviral production

HEK293T (passage <40) cells were cultured in MEM/ 5% FCS on Rx300 flasks till confluence. The media was removed and cells were infected with diluted adenovirus (1:9 in PBS/2% FCS). The infection assay was performed for 1 h at RT on a shaker. Afterwards 70 ml media was supplied to the cells. Infection was continued for 2 days at 37°C/95% humidity/5% CO_2.

7.4.3 Cell harvesting and virus purification

The infected cells were harvested from an Rx300 flask by using a cell scraper and subsequently transferred to 50 ml falcon tubes. Falcon tubes were centrifuged at 1500 rpm for 10 min. at 4°C and supernatants were discarded. Cell pellets from different flasks were pooled and finally taken up in 45 ml Tris-buffer (pH 8, 4°C). Cells were centrifuged (1500 rpm, for 10 min. at 4°C) and lysed by 3 freeze-thaw cycles. The resulting lysate was supplemented with 1/10 volume of Na-desoxycholate (5%) and incubated for 30 min. on ice. Afterwards, 1-2 µl benzonase were added to the lysate and incubated for 30-90 min. at RT. Sedimentation of cell debris was achieved by centrifugation for 10 min. 2500 rpm at 4°C. The resulting supernatant (containing viral particles) was removed and the sediment was frozen for possible re-isolaion of virus. The supernatant was refilled with Tris-buffer (0,1 M; pH 8) to exactly 3,1 ml. Furthermore, 1,8 ml saturated CsCl solution was added to the supernatant and mixed gently. The solution was transferred to an ultra-centrifugation tube. The tube was placed into an ultracentrifuge and gradients were centrifuged at 47000 rpm for 20 h. The band which corresponds to the virus was removed using a syringe and purified a second time using a CsCl gradient for 20 h. Afterwards, the volume of the virus containing solution was determined using a microliter pipette and the virus titrated.

7.4.4 Adenoviral infection of mice

Adenovirus was demonstrated to have hepatotropic capabilities (Huang *et al.*, 2012; Stabenow *et al.*, 2010) but does not replicate within hepatocytes if the *E1* encoding region is deleted. To generate an endogenous immune response against Ova within the mice, adenoviral infection was performed coincidental to Tam driven induction of Ova expression. Per mouse 1 x 10^{10} pfu Adeno-Luc-GFP (6.13 Viruses) were administered intravenously (i.v.) similar as described for adoptive transfers. To achieve hepatocyte specific expression of Ova coincidental to infection, mice were treated with Tam on day -1 and 0 and infection was performed on day 0. To track the route of infection, *in vivo* bioluminescence imaging (7.5.1.2 *In vivo* bioluminescence imaging) was performed on several time points relative to infection.

7.5 Work with mice

7.5.1 Application methods

7.5.1.1 Tamoxifen administration to mice

Tamoxifen was administered orally to the mice by a gavage, using a special feeding needle (Heiland Vet GmbH). To prepare a total concentration of 50 µg or 1 mg/ 400 µl, a Tamoxifen tablet (10 mg, Ratiopharm) was crushed into fine powder using a mortar and pestle. The powder was transferred into a falcon tube and supplied with 2 ml ClinOleic infusion solution, to get a stock concentration of 5 mg/ml. This stock solution was rotated at 4°C for at least 4 h. Afterwards the solution was diluted in ClinOleic (Baxter) to a final concentration of either 50 µg, 1 mg or 8 mg/400 µl and given to the mice. A single dose of 50 µg Tam turned out to be sufficient for the induction of *ova* sense orientation.

7.5.1.2 *In vivo* bioluminescence imaging

In vivo bioluminescence imaging is a powerful non-invasive tool to analyze *in vivo* kinetics without prior euthanization of experimental animals. In this work *in vivo* measurement of bioluminescence was used to determine the site of viral tissue tropism upon infection of mice with viruses, expressing the reporter gene *luciferase* (Tab. 15) using the Xenogen IVIS 200 system (Caliper Life Sciences). This system allows the detection of low light levels and is optimized for *in vivo* imaging (whole animal studies). Photons produced in the subject diffuse

through the tissues, the means of excitation at the surface of the imaged subject are captured by a sensitive photon charged-coupled device (CCD) camera.

For analysis of mice with this technique, the mice were first anaesthetized in a special chamber (Caliper Life Sciences) which provides 2-2,5% Isofluran (Allbrecht). Once the mice were anaesthetized, 100 µl luciferin (30 mg/ml in PBS, Synchem OHG) were administered intraperitoneally (i.p.). Afterwards, the mice were placed into the acquisition chamber immediately, as well providing 2-2.5% Isoflurane. To allow a systemic diffusion of the injected substrate, the measurement was started 4 min. after luciferin injection. To acquire the bioluminescent images, several parameters have to be adjusted to control the sensitivity and resolution of the signal:

- Field of view: depends on the distance between subject and camera lens
- Exposure time: the collection of emitted light is directly proportional to the amount of time the camera shutter is open
- Aperature (f/stop): controls the amount of light collected
- Binning: refers to the CCD resolution

7.5.1.3 Intravenous application

Intravenous (i.v.) application was always performed to administer viruses, pure CD8+ T cells or total splenocytes to recipient mice. Recipient mice were exposed to red light, to induce dilatation of the veins for ~4 min. prior to application. Mice were fixed within a restrainer and substrates were injected into the tail vein by a 26 gauge syringe (Braun) in the indicated volume (see chapters: 7.4.4 Adenoviral infection of mice, 7.7.3 *In vivo* cytotoxic T lymphocyte (CTL) assay and 7.7.4 Magnetic isolation of splenocytes).

7.5.1.4 Blood sampling and alanine amino transferase (ALT) measurement

Blood sampling was performed to indicate the liver function by measurement of serum ALT levels. Mice were anaesthetized by Isoflurane and blood was taken by retro-orbital bleeding, using a heparin coated capillary. Blood samples were added to 25 µl heparin (1,25 I.E, Ratiopharm) to avoid clotting. Samples were centrifuged at 13000 rpm for 5 min. and 32 µl plasma were loaded to a test strip (Roche Diagnostics, Mannheim) to measure the ALT concentration on a Reflovet®Plus reader (Roche Diagnostics, Mannheim).

7.6 Flow cytometry

A flow cytometer is a physical device that enables the investigation and examination of small particles, as for example cells. The method is based on a stream of fluid containing suspended cells. This stream creates a laminar flow and passes through a light beam, where an electronically detection apparatus is provided within the device itself. According to the laminar flow, cells pass the detector one by one (Brown & Wittwer, 2000). The apparatus enables both, detection and discrimination of cells according to their size and morphology. One detector is in line with the light beam and measures the size of passing particles (forward scatter (FSC)). Another detector is aligned perpendicularly to the FSC-detector and measures the granularity of passing cells (side scatter (SSC)) (Jin *et al.*, 2007). Since every cell type has a specific size and granularity, the correlation of FSC vs. SSC allows the discrimination of specific cells within a heterogeneous population. By adjusting the voltage during acquisition the resolution can be increased or decreased to visualize the target cells.

Flow cytometry allows simultaneous investigation of multiple parameters. Apart from discriminating different cell populations, phenotypic variations within one population can be investigated on <2000/ sec. (Brown & Wittwer, 2000). This is mediated by fluorescent dyes that can be applied to cell cultures or fluorescent dyes that are conjugated to specific antibodies. Antibodies bind to target epitopes present on the cells of interest and thus label the cells specifically. Since flow cytometers are provided with different lasers and filters, the cells are excited to emit light of a specific wavelength while passing the detection apparatus. The fluorescent dye emits a longer wavelength which can be detected by the filter (Brown & Wittwer, 2000). For each fluorescent emission peak, one filter is provided. By this combination characteristics of individual cells can be quantified.

In addition to phenotypic analysis, specific cell sorters are present which allow extrapolation of the cells of interest from a heterogeneous cell population. The principle is similar as described above and is also performed by surface staining. However, it has to be considered for which purposes sorted cells are required and which phenotype the cells should display upon sorting. If for example markers that induce cell activation are used during the sorting, the resulting cell fraction is not naïve any more.

Flow cytometry was used within this work to investigate the phenotype of leucocytes from various murine tissues upon immune responses. Moreover, cells were often analyzed for the expression of different markers prior to adoptive transfer experiments to investigate the phenotype of transplanted cells. For cellular acquisition the flow cytometer LSR II (BD) was used. Since multiple color stainings were performed, aliquots of cells were stained

individually with every dye used in the respective protocol to enable compensation. This is required to avoid cross emissions by different dyes that might lead to false positive signals within the multi-color stained samples.

7.7 Eukaryotic cell culture

For the work with eukaryotic cells only sterile pipettes, buffer, media, bottles and tubes were used. Work was done under sterile conditions using clean benches.

7.7.1 Isolation of immune cells from murine tissue

Mice were euthanized prior to cell isolation from any tissue. If the blood system had to be accessible, the mice were euthanized by an overdose of Isoflurane (Allbrecht GmbH). In case only specific tissues, e.g. spleens, were of interest, the euthanization could be performed by decapitation. Work was done under sterile conditions, using autoclaved surgical tools.

7.7.1.1 Isolation of primary murine lymphocytes from liver draining lymph nodes

The abdomen of previously euthanized mice was opened and the celiac lymph node (cLN) located close to the vena cava was isolated by using a small forceps. Afterwards, the cLN was transferred to a 100 µm cell strainer which was placed into a six well dish. 2-3 ml PBS/1% FCS were added to each well and the cLN was smashed through the cell strainer using a piston. The piston and cell strainer were washed twice with 1 ml PBS/1% FCS and the suspension transferred to a 15 ml falcon tube. After centrifugation, at 1500 rpm for 5 min., the supernatant was discarded and the cells resuspended in 1 ml PBS/1% FCS. Afterwards, the cells could be used for staining (see chapters: 7.7.5 Staining protocols for flow cytometry, 7.7.5.1 Surface staining of lymphocytes and 7.7.5.3 Intracellular FoxP3 staining).

7.7.1.2 Isolation of primary murine lymphocytes from the spleen

Splenic located lymphocytes were isolated for phenotyping of CD8+ T cells in final experiments. Alternatively, lymphocytes were isolated from spleens of BL/6 or OT-I mice if required for adoptive transfer experiments.

Mice were euthanized with an overdose of Isofluran (for T cell phenotyping) or decapitation (for adoptive transfer experiments). Using sterile scissors the left flank of the mice was opened and the spleen isolated. The spleen was transferred into 10 ml PBS/1% FCS and minced through a previously boiled steel sieve using a 5 ml syringe. The suspension was transferred into a 15 ml centrifugation tube and pelleted at 1500 rpm for 5 min. The supernatant was discarded and the pellet resuspended in 10 ml erythrocyte lysis buffer. The cells were incubated for 5 min. at room temperature and centrifuged again at 1500 rpm for 5 min. Following centrifugation, the pellet was washed twice in 10 ml PBS/1% FCS. Upon the last washing step, the suspension was transferred onto a cell strainer (100 µm) which was placed in a 50 ml falcon tube to remove fatty debris. For T cell phenotyping, the isolated splenocytes, containing lymphocytes were directly used for counting (see chapters: 7.7.2 Counting of isolated primary lymphocytes) or specific stainings (see chapters: 7.7.5 Staining protocols for flow cytometry, 7.7.5.1 Surface staining of lymphocytes and 7.7.5.3 Intracellular FoxP3 staining).

7.7.1.3 Isolation of primary murine lymphocytes from the liver

Upon finalizing an experiment, intrahepatic lymphocytes were isolated for phenotyping or RNA-sequencing analysis.

Mice were euthanized by applying an overdose of Isoflurane. Decapitation is not possible for this procedure, as the blood system needs to stay intact. Euthanized mice were fixed and the abdomen was opened. If blood samples for ALT or cytokine profiling were required, approximately 200 µl blood were taken from the vena cava or the heart and mixed with 25 µl heparin (1,25 I.E). A sterile butterfly needle was inserted into the vena cava and the liver was perfused with a total volume of 10 ml liver perfusion medium. During the perfusion, the portal vein was cut to enable blood flow. In case liver tissue had to be sampled for RNA-isolation (see chapter: 7.2.2.1 RNA isolation from liver tissue), small pieces of the liver were isolated during the perfusion procedure, transferred to a cryo vial which was directly stored in liquid nitrogen. For long-term storage, samples were always kept at -70°C. Upon perfusion, another syringe, containing liver digest medium, was connected to the butterfly needle and a

total volume of 5 ml was injected into the mouse. This enables in situ pre-digestion of the liver. Eventually, the gall bladder was removed and the whole liver transferred to a 50 ml falcon tube containing 10 ml liver digest medium. Following digestion of the liver, pieces of liver tissue were incubated in liver digest medium for 30 min. at 37°C which allows complete digestion of the tissue. Afterwards, the tissue was minced through a cell strainer by using a piston. The cell suspension was transferred to a 50 ml falcon tube (labeled with `A`) and filled with PBS to a total volume of 45 ml. To separate the hepatocytes from the non-parenchymal cells (NPC) which were the cells of interest, the samples were centrifuged at 500 rpm for 5 min. This allows pelleting of the hepatocytes, whereas the NPC fraction remains within the supernatant. The supernatant was transferred to a new 50 ml falcon (labeled `NPC`) and centrifuged again at 1500 rpm for 5 min. In addition, the hepatocyte pellet (in tube `A`) was filled with PBS to a total volume of 45 ml and centrifuged as described before. The supernatant in the tube labeled NPC was discarded. After the second fractionation of tube `A`, the NPC fraction from tube `A` was transferred to the pelleted NPC fraction from the first centrifugation. The NPC were centrifuged again at 1500 rpm for 5 min. The supernatant was discarded and the pellet resuspended in 3ml PBS/1% FCS. Then, 3,5 ml of a 70% percoll solution were added to the sample and the NPC cells were resuspended. Afterwards the cell suspension was gently overlaid on 70% percoll in a 15 ml falcon tube, creating a gradient. To separate the NPC, the gradient was centrifuged for 20 min. at 2000 rpm without brake to avoid excessive deceleration which would mix the different phases. Afterwards, the interphase, containing the lymphocytes, was aspirated using a Pasteur pipette and transferred to a 50 ml falcon tube preloaded with 20 ml PBS. To remove remaining percoll the cells were centrifuged at 1500 rpm for 10 min. (with brake). The supernatant was discarded and the cells resuspended in 10 ml erythrocyte lysis buffer, incubated for 5 min. at RT and centrifuged for 5 min. at 1500 rpm. Following removal of the supernatant, the cells were washed once with 10 ml PBS/1% FCS and centrifuged as described above. After this washing step, the supernatant was discarded again and the cells resuspended in 1 to 2 ml PBS/1% FCS, depending on the required volume. Lymphocytes could directly be used for counting and specific staining (see chapters: 7.7.2 Counting of isolated primary lymphocytes, 7.7.5 Staining protocols for flow cytometry, 7.7.5.1 Surface staining of lymphocytes and 7.7.5.3 Intracellular FoxP3 staining).

7.7.2 Counting of isolated primary lymphocytes

Isolated lymphocytes were counted either manually using a Fuchs-Rosenthal counting chamber or automatically using the Guava cell acquisition system (Merck Millipore). For

manual counting, 10 µl cell suspension were diluted with 90 µl Trypanblue to discriminate viable from dead cells. Following enumeration of cells within 3 separate squares (4 x 4 each) of the counting chamber, the mean was multiplied with the dilution factor, the chamber factor (5 for Fuchs-Rosenthal) and the total volume to calculate the total cell number. Manual counting was always done prior to adoptive transfer experiments, including *in vivo* CTL.

For automatic counting, 40 µl cell suspension were diluted 1:10 in PBS/1% FCS and measured with the guava cell acquisition system (Merck, Millipore). The settings were adjusted, using FSC vs. SSC. The concentration of lymphocytes was calculated automatically by the system and was depicted in cells/ml.

7.7.3 *In vivo* cytotoxic T lymphocyte (CTL) assay

Upon successful clearance of an acute infection, a minor fraction of antigen specific CD8+ T cells develops a pool of memory cells (Obar et al., 2011). These cells differ from naïve cells, as they are more abundant, have migratory capacity and can be reactivated by lower amounts of antigen to perform specific killing of an antigen presenting target cell (Mueller et al., 2013). According to those capabilities, antigen clearance upon adoptive transfer should establish a T cell population which is potent in reacting against reoccurring antigen in vivo. To investigate the presence of those cells, an in vivo cytotoxic T lymphocyte assay was applied. In this assay, a mixture of peptide loaded (pulsed) target cells and unpulsed control cells labeled with different concentrations of a fluorescent dye, were transferred into recipient mice. A specific killing of target cells by memory cells can be analyzed by flow cytometry (Aichele et al., 1997). Lymphocytes from the spleen were isolated as described above (see chapter: 7.7.1.2 Isolation of primary murine lymphocytes from the spleen) and re-suspendend in 20 ml RPMI medium. The cell suspension was divided equally into two separate tubes. Both tubes were refilled with RPMI medium to a total volume of 20 ml. The cells in one falcon tube were pulsed with target peptide by addition of 10 µg/ml Ova (Stock: 10 mg/ml). The other cells remained unpulsed. Both cell fractions were incubated at 37°C, 5% CO2 and maximal humidity of 95% for 45 min. Afterwards, both falcon tubes were filled with PBS to a total volume of 45 ml and centrifuged for 5 min. at 1500 rpm. The cells were washed again and resuspended in 5 ml PBS. The fraction which was pulsed with Ova was stained by addition of 0.69 mM CFSE to the cells, resulting in the CFSE high (CFSEhi) fraction. The unpulsed fraction was stained by 0.069 mM CFSE and thus labeled CFSE low (CFSElo). The cells were incubated for 10 min. at 37°C, 5% CO2 in the dark followed by supplementation with 10 ml RPMI medium. Then, the cells were incubated on ice for 5 min.,

washed twice with PBS, counted and adjusted to 2 x 107 cells per 100 µl in PBS. CFSEhi and CFSElo cells were mixed in a 1:1 ratio and transferred to recipient mice by intravenous (i.v.) injection. To avoid unspecific immune reaction due to sex specific gene expression, male recipients only received cells from male donors and female recipients always received cells from female donors.

The recipient mice were sacrificed 16 h upon transfer and the frequency of $CFSE^{hi}$ and $CFSE^{lo}$ cells in the spleens was analyzed by flow cytometry using the LSRII (BD).

To estimate the ratio of transferred $CFSE^{hi}$ vs. $CFSE^{lo}$ cells before each transfer, an aliquot of 100 µl was analyzed by flow cytometry using the LSRII (BD).

7.7.4 Magnetic isolation of splenocytes

Splenocytes were isolated as described above in chapter 7.7.1.2 Isolation of primary murine lymphocytes from the spleen.

For adoptive transfer experiments, the cells were counted using a Fuchs-Rosenthal (0,2 µm) or a Neubauer-Improved (0,1 µm) chamber and subsequently purified with a MACS $CD8a^+$ T cell isolation kit (Miltenyi) according to manufacturer´s instructions. Briefly, the cells were adjusted to a concentration of 10^7 cells/40 µl PBS/1% FCS and supplemented with 10 µl biotin-antibody cocktail/10^7 cells. The suspension was mixed and incubated at 4°C for 10 min. Upon incubation 30 µl buffer (PBS/1% FCS) and 20 µl anti-biotin-microbeads per 10^7 cells were added. The cells were incubated for additional 15 min. at 4°C. The cells were washed by refilling the tube to 50 ml with PBS/1% FCS and centrifuged at 1500 rpm for 5 min. Following washing, the cells were resuspended in 500 µl PBS/1% FCS per 10^8 cells and loaded onto an equilibrated LS-column (Miltenyi) for magnetic negative separation. Afterwards, the column was washed three times with 3 ml PBS/1% FCS. The flow-through (containing the negatively selected CD8+ T cells) was centrifuged at 1500 rpm for 5 min. and adjusted to a concentration of 5 x 10^6 cells/200 µl PBS. The cells were then transplanted into respective recipient mice by i.v. injection. Adoptive transfers were always performed with respect to sex specific differences. Thus, only OT-I cells isolated from males, were transplanted into males, while OT-I from females were transplanted into female recipients.

7.7.5 Staining protocols for flow cytometry

7.7.5.1 Surface staining of lymphocytes

To analyze the phenotype of immune cells isolated from liver, spleen and other lymphatic tissues, these were stained with different fluorescently labeled primary antibodies (AB) directed against a given molecule as specified in (Tab**.** 4). Per staining assay, 200 µl of the isolated cells from each tissue were transferred to a v-shaped 96-well microtiter plate and centrifuged at 1500 rpm for 5 min. The supernatant was discarded and the cells were resuspended in 150 µl FACS buffer (PBS/2% FCS) supplemented with 6 µg/ml FC-block to avoid unspecific interactions between the immune cells and the staining antibodies. Then, the cells were incubated for 10 to 15 min. at 4°C, centrifuged as described above and the supernatant discarded. 50 µl of a previously mixed staining solution, containing the AB of interest, were added to each well and the cells were incubated at 4°C for 30 to 60 min. Afterwards, each well was filled with 200 µl FACS buffer and the cells were centrifuged for 5 min. at 1500 rpm. After removal of the supernatant, the cells were washed twice with FACS buffer and resuspended in 150µl FACS buffer. Prior acquisition at the LSRII cytometer (BD), the cell suspensions were transferred into a conical FACS tube, containing 150 µl preloaded FACS buffer.

7.7.5.2 Cultivation and peptide stimulation of primary murine lymphocytes

To investigate the potential of murine immune cells to produce effector cytokines upon intrahepatic immune responses, isolated cells from liver and spleen were cultivated *in vitro* in the presence of the target peptide. The cultivation was performed in RPMI medium (5% FCS, 1% Pen/Strep, 1% Glutamine) in a cell incubator at 37°C, 5% CO_2 and 95% humidity. Freshly isolated immune cells were adjusted in RPMI to a concentration of 1 x 10^6 cells/ml if isolated from the liver. Cells isolated from spleens were adjusted to a concentration of 5 x 10^6 cells/ml RPMI. 5 x 100 µl of each cell suspension were seeded into a 96-well plate (for liver cells a U-shaped well plate was used, splenocytes were cultured in a flat bottom well plate). To specifically re-stimulate Ova specific CD8+ T cells, the culture medium was supplemented with 2,5 µg SIINFEKL peptide/ml (Stock: 100 mg/ml). As a positive control a duplicate of cells was stimulated unspecifically using 10 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 µg/ml ionomycin. 2 h upon cultivation, the splenocytes were transferred to a v-shaped microtiter plate. Splenocytes and liver cells were centrifuged at 1500 rpm for 5 min., the supernatant was discarded and the cells were resuspended in RPMI supplemented with either 2,5 µg/ml SIINFEKL and 3 µg/ml Brefeldin A or 10 ng/ml PMA, 1 µg/ml ionomycin and

3 µg/ml Brefeldin A. Brefeldin A was added to impair secretion of cytokines, as this chemical blocks the transport of vesicles from the ER to the Golgi apparatus. Cells were cultured in the presence of both, respective stimuli and Brefeldin A at 37°C for a minimum of 5 and a maximum of 7 h and then transferred to 4°C or directly processed. The surface staining was performed as described before (see chapters: 7.7.5 Staining protocols for flow cytometry

7.7.5.1 Surface staining of lymphocytes). After removing unbound AB by washing, the cells were fixed and permeabilized by resuspension in 100 µl Cytofix/Cytoperm™ (BD)/well followed by an incubation for 20 min. at 4°C in the dark. Permeabilized cells were washed twice with BD Perm/Wash™ buffer (diluted 1:10 in ddH_2O) which was also used as AB diluent. Finally, the cells were incubated for a minimum of 30 min. in the presence of the AB against the respective cytokine at 4°C and washed twice with FACS buffer. Cells were resuspended in 150 µl FACS buffer and analyzed by flow cytometry using the LSRII (BD).

7.7.5.3 Intracellular FoxP3 staining

The expression of the transcription factor FoxP3 has been shown to be exclusive for CD4+ regulatory T cells (T_{reg}) (Fontenot *et al.*, 2003; Fontenot & Rudensky, 2005). To gain insight into the regulative capacities of those cells during hepatitis or development of autoregulative cells in OT-II x Ova x Cre mice, those cells were also analyzed. Isolated lymphocytes (for more information see chapters: 7.7.1.1 Isolation of primary murine lymphocytes from liver draining lymph nodes, 7.7.1.2 Isolation of primary murine lymphocytes from the spleen and 7.7.1.3 Isolation of primary murine lymphocytes from the liver) were stained first with the LIVE/DEAD® Fixable Near IR Dead cell stain kit (Invitrogen). This excludes false positive signals originating from dead cells as they are prone to bind antibodies unspecifically. Cells were washed with PBS and stained with 50 µl solution (1:500 Live/Dead stain in PBS) for 30 min. at 4°C. Afterwards, the cells were washed with FACS buffer and stained with antibodies against the surface markers CD4, CD8, CD25 as described above (7.7.5 Staining protocols for flow cytometry and 7.7.5.1 Surface staining of lymphocytes). To specifically label the intracellular located transcription factor FoxP3, cells were resuspended in 200 µl of a freshly prepared fixation/permeabilization working solution (eBioscience) and incubated at 4°C for 1 to 18 h. Afterwards, the samples were washed with 400 µl permeabilization buffer and then further incubated for 15 min. at 4°C in 50 µl permeabilization buffer, containing 40 µg/ml rat IgG which was used as an intracellular blocking reagent. A PE-conjugated anti-FoxP3 AB was diluted 1:250 in permeabilization buffer. 50 µl of this antibody solution were added to the permeabilized cells and they were incubated for additional 30 min. at 4°C.

Afterwards, the cells were washed twice with 400 µl permeabilization buffer, resuspended in 150 µl FACS buffer and acquired on the LSRII (BD).

7.8 Statistical analysis

Data are represented as mean of biological replicates in groups of mice ± standard deviation (SD) if groups are n≥3. To compare the variation of two different data sets, Mann-Whitney U test was used if n≥4. Significant differences between sets are considered for p-values: * p≤0,05; ** p≤0,01; *** p≤0,001 and **** p≤0,0001.

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